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Spatial and temporal targeting of gene expression in *Drosophila* by means of a tetracycline-dependent transactivator system

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SUMMARY

In order to evaluate the efficiency of the tetracycline-regulated gene expression system in *Drosophila*, we have generated transgenic lines expressing a tetracycline-controlled transactivator protein (*tTA*), with specific expression patterns during embryonic and larval development. These lines were used to direct expression of a *tTA*-responsive promoter fused to the coding region of either the β-galactosidase or the homeotic protein Antennapedia (ANTP), under various conditions of tetracycline treatment. We found that expression of β-galactosidase can be efficiently inhibited in embryos and larvae with tetracycline provided in the food, and that a simple removal of the larvae from tetracycline exposure results in the induction of the enzyme in a time- and concentration-dependent manner. Similar treatments can be used to prevent the lethality associated with the ectopic expression of ANTP in embryos and, subsequently, to control the timing of expression of the homeoprotein ANTP specifically in the antennal imaginal disc.

Our results show that the expression of a gene placed under the control of a tetracycline-responsive promoter can be tightly controlled, both spatially by the regulatory sequences driving the expression of *tTA* and temporally by tetracycline. This provides the basis of a versatile binary system for controlling gene expression in *Drosophila*, with an additional level of regulation as compared to the general method using the yeast transcription factor GAL4.

Key words: Tetracycline, Gene expression, *Drosophila*, *Antennapedia*

INTRODUCTION

An essential and general experimental approach to analyse the function of a gene in a whole organism is to examine the phenotypic consequences of its directed expression in certain cells, or at a developmental stage, in which the gene is normally silent. In *Drosophila*, two major systems have been designed to achieve the conditional expression of gene constructs integrated into the genome. In the first, the coding sequence of the gene of interest is placed under the control of promoters inducible according to the culture conditions. The *hsp70* gene promoter is commonly use for that purpose (see Schneuwly et al., 1987 and references therein). High levels of induction can be obtained at well-defined time periods during development upon exposure of the organism to elevated temperatures. This advantage is often limited because ectopic expression occurs in all cells and endogenous genes are repressed during heat shocks. Consequently, side defects including lethality may mask the result of the ectopic expression in the desired cell type(s). It is also frequently necessary to repeat heat shocks over an extended time period to observe the phenotypic consequences, and it may be difficult to distinguish between the primary and secondary effects of the overexpression (e.g. Gibson and Gehring, 1988).

An alternative approach is to engineer a gene construct inducible by a single transcription factor whose activity can be controlled *in vivo*. The ability of the yeast transcription factor GAL4 to activate transcription in *Drosophila* (Fisher et al., 1988) has been exploited to generate a versatile method for targeting gene expression in this organism (Brand and Perrimon, 1993). Any gene of interest can be placed under the control of a promoter containing GAL4 upstream activating sequences (UAS) and integrated stably into the genome of a parental line (responder strain), since it remains silent in the absence of GAL4. Tissue-specific expression of the gene is obtained upon crossing to a second parental line (driver strain) expressing the transcription factor under the control of a suitable promoter. Although a large number of strains expressing GAL4 in a wide variety of patterns can be selected on the basis of the expression of a reporter gene bearing the UAS sequences (Brand and Perrimon, 1993; Yeh et al., 1995; Calleja et al., 1996), only a few of them have been used to direct expression of functional proteins in the post-embryonic stages of development (Brand and Perrimon, 1993; Capdevila and Guerrero, 1994; Hinz et al., 1994; Speicher et al., 1994; Rimmington et al., 1994; Ferver et al., 1995; Halder et al., 1995; Zink and Paro, 1995; Freeman, 1996; Morimura et al., 1996). The limit in this binary system lies in the lack of

temporal control, which remains primarily determined by the regulatory sequences driving GAL4. Because most of the gene-specific enhancers are active at various stages of development in *Drosophila*, GAL4-mediated induction is frequently observed from the embryonic stage onwards, and often results in premature lethality (e.g. Halder et al., 1995). To overcome this difficulty, Flp-mediated recombination has been used to achieve conditional expression of a transgene upon recombination of an FRT cassette separating the coding region from a promoter (Struhl and Basler, 1993). This approach can be used to control the expression of GAL4 (Pignoni and Zipursky, 1997). In any case it requires the combination of various specific constructs and it can be applied only to tissues in which cell division occurs, since it results in the generation of clones of expressing cells.

Extension of the more versatile binary system for post-embryonic studies could be achieved with the use of a regulatory protein modulated in the organism with an innocuous effector. One of the best candidates is the tetracycline-dependent transactivator (*tTA*) comprising the tetracycline repressor of *E. coli* (*tetR*) and the strong transcriptional activation domain of the herpes simplex virus protein VP16 (Gossen and Bujard, 1992). The high affinity and specific binding of *tetR* to the tetracycline operator sequences (*tetO*) can be inhibited by tetracycline (Hillen and Wissmann, 1989) and is thought to result from a conformational change of *tetR* upon association with tetracycline (Hinrichs et al., 1994). In HeLa cells, *tTA* was found to stimulate transcription of a promoter bearing a multimerized *tetO* by several orders of magnitude, and a fast and reversible switch of the *tTA*-dependent promoter was obtained upon addition or removal of tetracycline from the culture medium (Gossen and Bujard, 1992). These features have been extensively exploited in tissue culture where tetracycline levels can be tightly controlled. Tetracycline-regulated expression of reporter genes was also demonstrated in whole plants (Weinmann et al., 1994) and in transgenic mice (Hennighausen et al., 1995; Kistner et al., 1996). In the latter case, the efficiency of tetracycline, administrated by slow-release tetracycline pellets or in drinking water, has been mostly evaluated by analysing the expression of sensitive reporter genes, although a few examples of successful expression of proteins have been reported (see Shockett and Schatz, 1996 for a review).

In this study we determined the functional properties of *tTA* in *Drosophila* by expressing this regulatory protein under the control of various promoters. Using a *lacZ* reporter gene placed under the control of a promoter bearing seven copies of *tetO*, we found that expression of β-galactosidase can be tightly controlled in embryos and larval tissues. Furthermore, we used *tTA*-expressing strains and tetracycline treatments to drive tissue-specific ectopic expression of the homeoprotein *Antennapedia (Antp)* at different stages of development. Our results demonstrate the usefulness of the *tet* system in *Drosophila*.

MATERIALS AND METHODS

DNA constructs

Most of the constructs were assembled by multiple step cloning according to standard methods (Sambrook et al., 1989). Further details and maps are available upon request.

tTA driver constructs

hsp70-tTA

The *tTA* coding region was isolated as a 1.1 kb *Eco*RI-*Bam*HI fragment from pUHD 15-1 (Gossen and Bujard, 1992) and cloned into CaSpeR-hs (Thummel and Pirrotta, 1992)

RHT (*rosy*, *hsp70* promoter, *tTA*)

This P element vector derives from the enhancer-test vector HZ50PL (Hiromi et al., 1985). The *tTA* coding region is flanked by the minimal promoter and the poly(A) sequences of *hsp70* (Fig. 1A).

ey-tTA and *HoxA7-tTA*

The *eyeless (ey)* gene enhancer (a 3.5 kb *Kpn*I fragment from *ey* Eco 3.6) (B. Hanck, T. Eggert, W. J. Gehring and U. Walldorf, unpublished) and a 630 bp fragment of the intron of the *HoxA7* gene from pB6 (Haerry and Gehring, 1996) were cloned in RHT to give *ey-tTA* and *HoxA7-tTA*, respectively.

Tetracycline-responder constructs

tetO-lacZ

The heptameric repeat of the tet operator was isolated as a 310 bp *Eco*RI-*Kpn*I fragment from pUHC 13-3 (Gossen and Bujard, 1992) and cloned upstream of the P-*lacZ* fusion of the enhancer-test vector CPLZ (Wharton and Crews, 1993). CPLZ contains the P-element transposase promoter (up to –42 from the cap site) and the N-terminal transposase sequence fused in-frame with *lacZ* and the polyadenylation signal of *hsp70*.

WTP (white-*tetO*-P promoter)

This P-element vector was constructed to express any gene under the control of a tetracycline-responsive promoter. It contains the vector backbone of CPLZ, the heptameric repeat of the tet operator, the P-element promoter and leader sequences from Carnegie 4 (Rubin and Spradling, 1983) and the polyadenylation signal of SV40.

tetO-Antp and *tetO-AntpΔHD*

The cDNAs encoding a full-length ANTP protein or a variant with a deletion of the homeodomain were isolated as *Not*I fragments from pHSSΔA and pNHT-A11, respectively (Gibson et al., 1990) and cloned into the corresponding site of WTP.

Germline transformation and *Drosophila* strains

P-element mediated transformation of ry^{506} or *y ac* w^{1118} recipient strains was carried out essentially as described (Spradling, 1986). A description of the markers and balancer chromosomes indicated in Figs 3 and 4 can be found in Lindsley and Zimm (1996). A405.1 M2 and rK781 have been described (Wagner-Bernholz et al., 1991; Flister, 1991).

Tetracycline media and treatments

A tetracycline-containing medium suitable for larval feeding and maintenance of adults was obtained by mixing 100 ml of tetracycline solution (tetracycline hydrochloride (Sigma) diluted with sterile water at the required concentration), 25 g of Instant *Drosophila* Food (Carolina Biological Supply) and 1 g of dry yeast.

Tetracycline was provided to adult females by placing 50-70 virgins in a glass vial containing a 2.5 cm filter (Whatman grade 3 MM) soaked with 500 μl of a 4% sucrose solution with tetracycline at the appropriate dilution. Females were fed for 3-4 days, following a daily cycle of 16 hours on tetracycline-containing filters and 4 hours on standard food supplemented with a drop of yeast paste. Males of the relevant genotype were placed with females on tetracycline and allowed to mate overnight before the beginning of egg collections. For

experiments requiring larval feeding, batches of 100-200 eggs harvested from grape juice plates were placed on pieces of nylon mesh, and allowed to develop at 25°C on tetracycline-containing food. When necessary, larvae were separated from their food by floating in 30% glycerol, collected with forceps, washed with PBS and transferred on standard food in groups of 50 to 100.

Phenotypic analyses

Embryos and larval tissues were fixed and stained for β-galactosidase as described (Bellen et al., 1989). Adult heads were separated from the body of narcotised flies and holes were made into the cuticle to facilitate penetration of the fixative. For antibody staining of embryos and examination of cuticular phenotypes, standard procedures were applied (Ashburner, 1989).

RESULTS

We have designed a general system to express *tTA* under the control of regulatory sequences (RHT driver construct), in order to direct the expression of a gene of interest under the regulation of a tetracycline-responsive promoter (WTP responder construct). The gene constructs can be stably propagated into the genome of separate strains, and *tTA*-dependent gene induction is obtained in the F_1 offspring of the cross where it can be controlled by tetracycline (Fig. 1A).

tTA is a potent transactivator in *Drosophila*

In order to analyse the transactivation potential of *tTA*, we have used an indirect heat shock assay to drive ubiquitous expression of *Antp* in embryos. Heat shock assays were performed on embryos carrying *tTA* under the control of the *hsp70* gene promoter (*hsp70-tTA*) and a WTP derivative carrying a full-length *Antp* cDNA (*tetO-Antp*). Independent transformants were found to give an identical embryonic phenotype to the H4 line that carries a direct *hsp70-Antp* construct (Fig. 1B; see also Gibson and Gehring, 1988 for a complete description of the H4 line). In contrast, heat-shocked embryos carrying a WTP derivative with a deletion of the homeodomain (*tetO-Antp*ΔHD) or the empty WTP vector (*tetO–*) showed a wild-type cuticle and developed to the adult stage (Fig. 1B and data not shown). A western blot of embryonic extracts prepared from heat-shocked embryos and probed with an ANTP-specific monoclonal antibody (Condie et al., 1991) reveals similar levels of ANTP expression (Fig. 1C). In addition, transformation of the adult antenna into a mesothoracic leg can be obtained when heat shocks are applied to third instar larvae (Gibson and Gehring, 1988; D. Resendez-Perez, B. Bello and W. J. Gehring, unpublished). Taken together, these results show that *tTA* can activate transcription of a promoter that contains *tetO* sequences without any toxic effect of this regulatory protein in *Drosophila*.

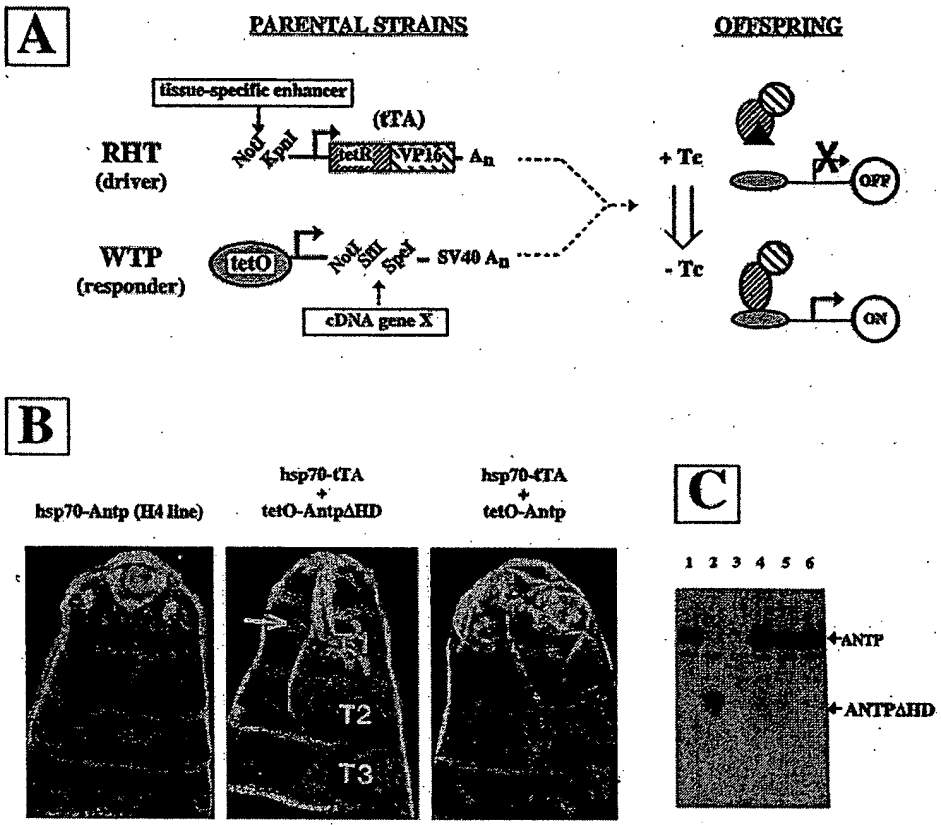

Fig. 1. Transgenic constructs and the transactivation potential of *tTA* in *Drosophila*. (A) Schematic representation of the tetracycline-inducible system in *Drosophila*. The transformation vectors RHT and WTP are, respectively, designed to express *tTA* under the control of enhancer-like elements and any cDNA under the regulation of a *tTA*-responsive promoter that contains *tetO* sequences. Derived constructs are integrated and propagated in two separate parental strains. In absence of tetracycline (–Tc), the binding of *tTA* results in the activation of gene X with the spatial and temporal specificities determined by the driver construct (on state). Raising the offspring on a tetracycline-containing medium is used to prevent the binding of *tTA* in order to keep the responder construct silent up to a certain point of time (off state). Stopping tetracycline exposure allows time-specific induction of gene X upon withdrawal of the antibiotic. (B) Cuticular preparations of embryos heat shocked for 30 minutes at 6.5 hours of embryogenesis. All embryos carry a single copy of the constructs indicated at the top. Note the similar defect in head evolution in *hsp70-Antp* and *hsp70-tTA*; *tetO-Antp*, and the T1 to T2 transformation when compared to the wild-type cuticle of *hsp70-tTA*; *tetO-Antp*ΔHD embryos. The identical segmental transformation is revealed by the disappearance of the characteristic denticle belt of the first thoracic segment (white arrow), indicating a transformation of T1 towards T2. The head segments are also transformed towards T2. T1, T2, T3: first, second and third thoracic segments respectively. (C) Detection of the ANTP proteins by western blot analysis in crude extracts of embryos heat shocked for 2 hours with a 4 hour recovery. Lane 1, *hsp70-Antp* embryos (H4 line); lanes 2- 6, embryos carrying *hsp70-tTA* and either *tetO-Antp* ΔHD (lane 2) or the empty WTP vector (lane 3), or independent insertions of *tetO-Antp* (lanes 4-6).

Tetracycline-controlled expression of *lacZ* during larval development

In order to express *tTA* during larval development, we inserted the eye-specific enhancer of the *ey* gene (Quiring et al., 1994) into the vector RHT and generated *ey-tTA* transformants. The expression of *tTA* was detected specifically in the eye imaginal disc by means of a *lacZ* reporter gene placed under the control of a promoter bearing *tetO* sequences (Fig. 2). Detection of β-galactosidase activity in the eye disc of the larvae reveals two essential features. First, enzymatic activity is detectable within less than 15 minutes of incubation with X-gal revealing a high level of expression of *tTA*. Second, this activity is detected in the eye disc over an extended period of development with a dynamic pattern (Fig. 2A, top row). In the early third instar, expression is detected uniformly in the eye part of the eye-antennal disc (left panel), which corresponds to the undifferentiated cells of the eye epithelium (Ready et al., 1976). The same pattern was observed in second instar larvae (not shown). Eye discs stained at different times during the third instar show that β-galactosidase activity gradually fades in the anterior region of the disc as the morphogenetic furrow moves anteriorly.

To determine whether tetracycline incorporated in the larval food could inactivate *tTA*, we stained early third instar larvae raised on media containing increasing concentrations of the antibiotic (Fig. 2A, time 0). No activity could be detected in the eye-disc of larvae exposed to as little as 0.1 μg/ml even after prolonged staining (24 hours) in X-gal solution (Fig. 2A, left column). Lower concentrations failed to inactivate lacZ expression (not shown). The results indicate an efficient uptake of tetracycline from the food and its diffusion to the imaginal discs, leading to a complete inactivation of *tTA* in a concentration-dependent manner. The same dose-response was obtained for larvae exposed to tetracycline for approximately 2 more days, suggesting that the concentration of tetracycline in the larval haemolymph does not change dramatically during the feeding period (not shown). In contrast, stopping the larval exposure to tetracycline was expected to decrease the level in the haemolymph and to restore the ability of *tTA* to bind and transactivate the *lacZ* reporter gene. To test this hypothesis, early third instar larvae were transferred to standard food, fixed and stained every 6 hours for β-gal activity (Fig. 2A). Enzymatic activity was detected 6 hours after the removal of the larvae from the medium containing 0.1 μg/ml tetracycline (Fig. 2A, second row from the top) or in a range of 18 to 24 hours when the larvae were exposed to 1 μg/ml or 10 μg/ml tetracycline, respectively (Fig. 2A, third and fourth row from the top). After induction, 12-24 hours were necessary to obtain

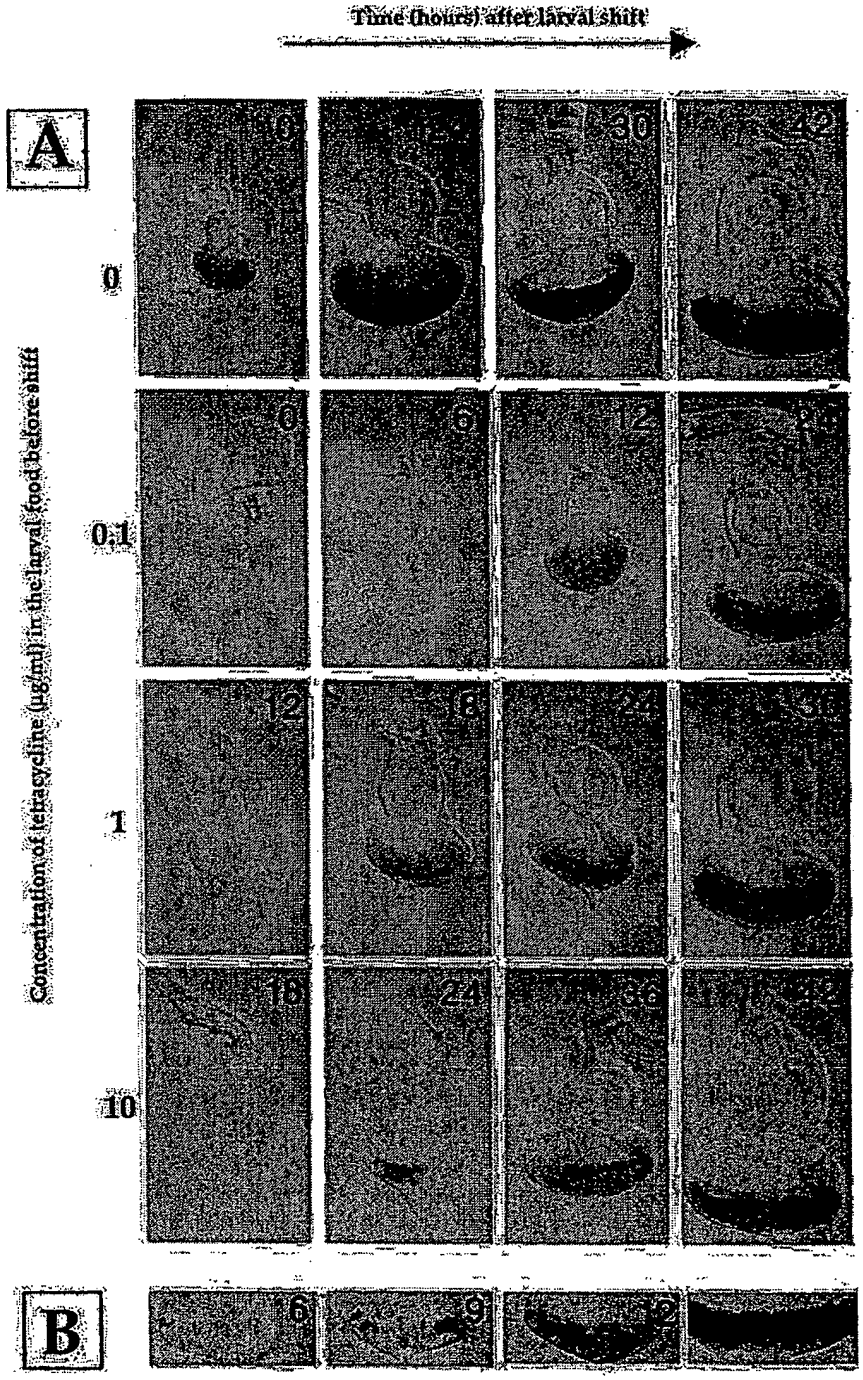

Fig. 2. Tetracycline controlled expression of *lacZ* in the eye imaginal disc driven by the *ey-tTA* strain. (A) Larvae of the genotype *ey-tTA/+; tetO-lacZ/+* were raised in the presence of increasing concentration of tetracycline, shifted to standard food at the early third instar and dissected at different times after shifting, indicated in hours at the top right of every figure. (B) Temporal profile of *lacZ* induction from larvae removed from 0.1 μg/ml tetracycline during the late third instar. Note the increasing number of cells expressing *lacZ*.

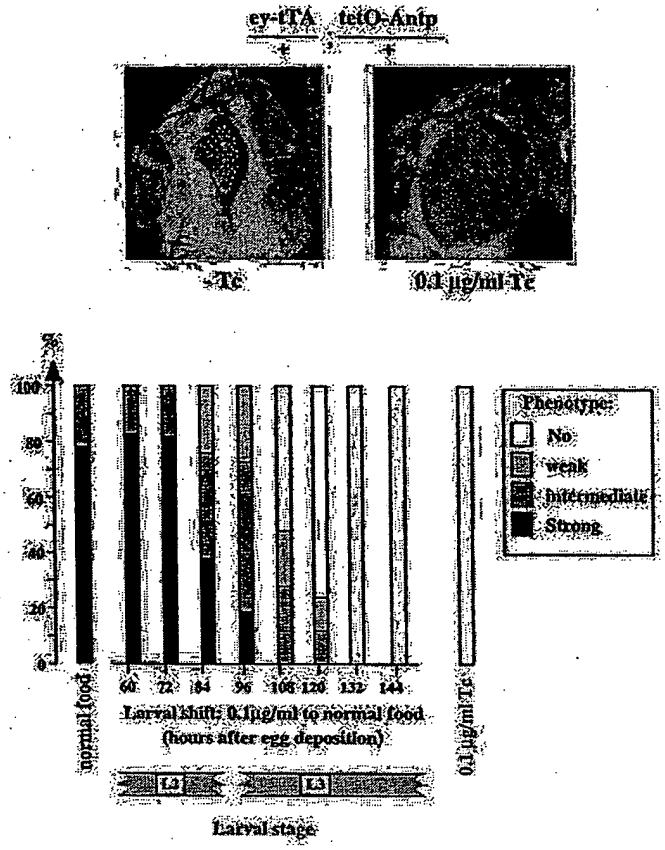

Fig. 3. Induction of *Antennapedia* in the eye imaginal disc by means of tetracycline. (Top) Eyes of adult flies raised in absence (–Tc) or in the presence of 0.1 µg/ml tetracycline (Tc) in the larval food. (Bottom) Semi-quantification of the eye phenotype in adults derived from larvae raised with 0.1 µg/ml tetracycline and shifted to standard food at 12 hour intervals. At least 50 adults were scored at every time point. Larval stages were identified on the basis of the morphology of the tracheal system and the anterior spiracles.

a comparable staining intensity in imaginal discs isolated from larvae exposed to tetracycline and control larvae. These data show that the withdrawal of the larvae from tetracycline is followed by a rapid induction of the *tetO-lacZ* transgene under the control of *tTA*. The concentration- and time-dependent expression of *lacZ* is likely to reflect the need to lower the concentration of tetracycline in the disc cells below a certain threshold level, which allows *tTA* to bind the tet operator and to stimulate transcription of the promoter. A careful examination of the staining patterns also suggested that the *tetO-lacZ* transgene was not turned on in every cell at the same time after withdrawal of tetracycline (Fig. 2A). To confirm this observation, we performed larval shifts during the second half of the third instar, when expression of *tTA* is uniform in the posterior part of the eye disc. Upon removal of the larvae from 0.1 µg/ml tetracycline, induction of *lacZ* can clearly be observed in a gradually increasing number of cells (Fig. 2B). Similar observations were obtained with different *tTA*-expressing strains, suggesting variations in the kinetics of the clearance of the antibiotic and/or the trancriptional activation.

Tetracycline-controlled expression of *Antennapedia* targeted to the eye disc

To determine the relevance of the data obtained with the *lacZ* reporter gene we used the same driver strain to direct expression of *Antp* under various conditions of tetracycline treatment. In the absence of tetracycline, adults obtained from

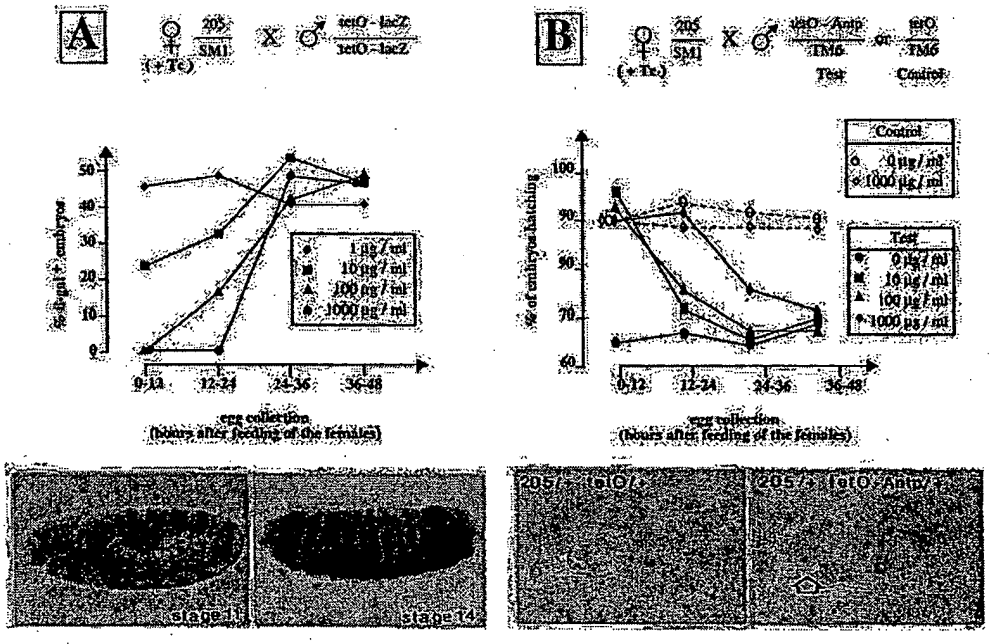

Fig. 4. Inhibition of *tTA*-dependent gene expression in embryos by maternal transmission of tetracycline. (A) Repression of the *lacZ* reporter driven by the 205 strain, shown at the bottom by immunodetection of β-galactosidase. Embryos collected over successive 12 hour periods following the feeding of 205/SM1 females with tetracycline, were stained for β-gal activity. At least 100 embryos older than the germ band extended stage (stage 11) were scored at every time point. 50% of transheterozygotes were expected in the offspring of the cross indicated at the top. (B) Tetracycline-dependent rescue of embryonic lethality by repression of the *tetO-Antp* transgene. Embryos collected over 12 hour periods after feeding of their mothers with tetracycline were allowed to develop at 25°C and the percentage of hatching larvae determined. At least 200 embryos were scored at every time point. 25% of transheterozygotes are expected from the cross indicated at the top. *tetO* refers to an insertion of the empty vector WTP on the third chromosome, used as a control. Bottom: cuticular phenotype of embryos of the indicated genotype. Note the failure of head involution marked by the open arrow in embryos expressing *Antp* under the control of the 205 strain.

a cross between the parental *ey-tTA* and *tetO-Antp* strains showed reduced and irregular compound eyes (Fig. 3, –Tc). Since *Antp* is normally not expressed in the eye disc (Wirz et al., 1986), its expression in this tissue would interfere by unknown mechanisms with the normal development of the eye. No other morphological defects could be detected in adults, in agreement with the eye-specific expression of *tTA* detected with the *lacZ* reporter. To ascertain that the eye phenotype resulted from the ectopic expression of *Antp* in the eye disc, we raised larvae on a medium containing tetracycline at various concentrations. The eyes were restored to a wild-type appearance with 0.1 μg/ml tetracycline (Fig. 3, top) but not with 0.01 μg/ml tetracycline, in good agreement with the dose-dependent repression of the *lacZ* reporter (Fig. 2A).

As indicated with the *lacZ* reporter, the directed expression of *Antp* by the *ey-tTA* strain should occur continuously throughout larval development and shift rapidly during the third instar when the cells undergo differentiation. We used tetracycline to control the timing of *Antp* overexpression in order to determine the functional significance of this dynamic pattern of expression, with respect to the alteration of the eye development. Newly hatched larvae were first fed with 0.1 μg/ml to inhibit *tTA* activity and then transferred to a standard medium every 12 hours to induce *Antp*. Adults were scored for eye defects and classified according to an arbitrary scale of strong, intermediate, weak or no detectable eye phenotype (Fig. 3, bottom). All the adults derived from larvae exposed to tetracycline up to the second instar showed strong eye defects in a range indistinguishable from their siblings raised in absence of tetracycline. In contrast, subsequent shifts allowing induction of *Antp* from the early third instar onward led rapidly to a complete rescue of the eye morphology. These results confirm the efficiency of the tetracycline control with a functional homeoprotein and suggest that the alteration in the normal eye development is mostly dependent on the ectopic expression of *Antp* in the undifferentiated cells of the eye epithelium.

Repression of *tTA*-dependent gene expression in embryos by maternal transmission of tetracycline

The embryonic development of *Drosophila* is not easily amenable to antibiotic treatment since the egg is protected by an impermeable set of eggshells but it is relatively fast (22-24 hours at 25°C) and maternal components are transmitted to the oocyte by the nurse cells and the follicle cells in the female ovaries. The influence of tetracycline given to the parental females was first tested on the strong *lacZ* expression driven by the *tTA* construct of the line 205 (Fig. 4A). This driver line 205 was isolated among twelve independent transformants of the *HoxA7-tTA* construct (see Materials and Methods) because of its unique expression pattern observed in the antennal disc (Fig. 5), the leg discs, the central nervous system, the epidermis and various internal tissues (not shown). Since the other lines showed a reproducible pattern in the eye disc and the larval brain due to the *HoxA7* enhancer (not shown), the line 205 is likely to reflect a modified expression of the transgene under the influence of genomic regulatory sequences flanking the integration site (Wilson et al., 1990). When assayed in embryos with the *lacZ* reporter gene, expression of *tTA* in the 205 strain starts at the end of germ band extension, about 5 hours after egg laying (AEL), and is detected mostly in the trunk region with a segmentally repeated pattern (Fig. 4A, bottom). This pattern changes rapidly, so that at the end of germ band retraction (approximately 10 hours AEL), strong expression is detected all over the ectoderm. The staining appears patchy in the cephalic segments and is not uniformly distributed in the thoracic and abdominal segments (Fig. 4A, bottom). After treatment of the females with tetracycline (see Materials and Methods), repression of the *lacZ* reporter is mostly effective in the eggs collected immediately after the end of exposure to the antibiotic and is dose-dependent (Fig. 4A, top). Repression was obtained in 100% of the eggs collected within 12 hours after the treatment of the females with 100 μg/ml or more of the antibiotic, and in a large fraction of them with 10 μg/ml. The gradual loss of repression observed in the eggs collected later is likely to reflect a decrease of the maternal pool of tetracycline accompanying the continuous production of eggs.

The same procedure of tetracycline treatments was tested for its effect on the survival rate of embryos carrying the driver construct 205 and either a *tetO-Antp*, or an empty responder construct (*tetO–*), as control (Fig. 4B). Examination of the embryonic cuticles revealed major defects in the formation of the head (Fig. 4B, bottom), a phenotype reminiscent of heat-shocked embryos in which *Antp* was ubiquitously expressed (Fig. 1B), although no homeotic segmental transformations were observed. This might reflect a different level of induction of *Antp* as compared to the use of a heat shock promoter but it is more likely to be due to a difference in the timing and the spatial expression of the homeoprotein, since transformations obtained by heat shocks are optimal when induced at 5-7 hours of development (Fig. 1B, see also Gibson and Gehring, 1988), at a stage when the 205 driver is mostly active in the trunk and is not ubiquitously expressed (Fig. 4A, bottom). Nevertheless, line 205 allowed us to test the effect of tetracycline on the survival rate of the embryos and, as shown in Fig. 4B, the embryonic lethality could be overcome in a dose-dependent manner by providing tetracycline to the females. The embryonic rescue was in good agreement with the tetracycline-mediated repression of the *tetO-lacZ* transgene (Fig. 4). These two independent assays clearly demonstrate the possibility to inactivate *tTA* in embryos in order to keep a promoter silent during this stage of development.

Targeted mis-expression of *Antp* in larvae following embryonic rescue

The efficient inactivation of *tTA* in embryos prompted us to analyse the fate of tetracycline-rescued embryos in more detail. As expected, embryos did not develop to the adult stage in the absence of tetracycline in the larval food, whereas addition of tetracycline led to the recovery of viable adults in a concentration-dependent manner (the quantitative data are available upon request). In addition, larvae raised under optimal conditions were shifted to standard food at various times to allow induction of *Antp*. Shifts performed during the late third instar led to the recovery of pharates or adults, whereas larvae shifted earlier essentially failed to undergo metamorphosis. Examination of the adults revealed very specific morphological modifications of the antennae and the head vertex (Fig. 5K-M), in the area expressing the *lacZ* reporter under the regulation of the 205 driver line (Fig. 5E-G). In contrast, transheterozygotes raised continuously with 10 μg/ml tetracycline showed wild-type structures (Fig. 5H-J),

demonstrating the highly specific alterations in the development of adult flies following the mis-directed expression of *Antp* by the 205 line. As revealed with the *lacZ* reporter, expression of *tTA* follows a dynamic spatial pattern in the primordia of the antenna from the mid-third instar onwards (Fig. 5A-D) and in the presumptive area of the occelli from the larval/pupal transition onward (Fig. 5C,D). Moreover, the lag in induction imposed by the removal of the larvae from tetracycline exposure suggests that the alterations of adult structures mostly result from the ectopic expression of *Antp* during the pupal stage. No defects were found in the legs or the palps where the 205 driver is also strongly expressed (not shown), in agreement with previous observations showing that only the derivatives of the eye-antennal disc respond to the ubiquitous expression of *Antp* induced by heat shock (Gibson and Gehring, 1988). These latter studies showed that repeated pulses of heat-shock expression are required during the third larval instar to achieve complete antenna to leg transformations. Our results confirm previous observations showing that the late larval induction of *Antp* does not induce fully differentiated morphological markers of the leg (Scanga et al., 1995; Larsen et al., 1996). Although we observe different arrangements of bristles on the antenna, none of them showed the bracts characteristic for leg bristles.

Directed expression of *Antp* in the antennal disc by *tTA* activates *rK781*

Since the observation of adult phenotype required late larval shifts, we asked whether the consequences of *tTA*-dependent expression of *Antp* could be directly assayed in the imaginal discs. As a marker, we used the enhancer detector line rK781, which was isolated in a screen for *Antp*-regulated genes on the basis of their response to the overexpression of the ANTP homeoprotein in the eye-antennal disc (Wagner-Bernholz et al., 1991). We combined driver, responder and test constructs in larvae, exposed them to tetracycline treatment, and assayed β-galactosidase expression in wandering third instar larvae. When raised continuously with 10 μg/ml tetracycline, the normal pattern of rK781 expression was detected in all the discs (not shown) and in a few cells of the antennal disc (Fig. 6, left), as previously described (Wagner-Bernholz et al., 1991; Flister, 1991). When dissected from larvae that were removed from tetracycline exposure, *lacZ* expression could be reproducibly detected in the form of a crescent at the border between the arista and the third antennal segment (Fig. 6, middle). This area corresponds to the most proximal part of the wedge-shaped sector expressing *tTA*, as visualised with the *lacZ* reporter (Fig. 6, right) and is also the first to express *tTA* during third instar (Fig. 5B). These results show that derepression of *Antp* by removal of tetracycline can be demonstrated by the activation of a downstream target gene in the antennal disc. These findings indicate that the tetracycline-dependent expression system efficiently repressed *Antp* in embryos and allows subsequent derepression in imaginal discs.

DISCUSSION

In this study, we report a detailed evaluation of the different properties of the tetracycline-dependent gene expression system in *Drosophila*. Since its description in transformed HeLa cells (Gossen and Bujard, 1992) this regulatory system has been extensively used in cell culture. In higher eucaryotes including plants and mouse, tetracycline-controlled activity of *tTA* has been mostly evaluated on reporter genes (see Shockett and Schatz, 1996 for a review), although a few examples of successful expression of proteins have been reported (Efrat et al., 1995; Ewald et al., 1996; Mayford et al., 1996; Shockett et al., 1995; St-Onge et al., 1996). By using *Drosophila* lines expressing high levels of *tTA*, we show that the strong induction of the *lacZ* reporter can be efficiently prevented by tetracycline in both embryos and imaginal discs. We have evaluated the dose-response and defined easy and reliable protocols of tetracycline treatment to control the repression of the *lacZ* reporter gene. Furthermore, we also show that this system is fully functional to control the spatial and temporal expression of the ANTP homeoprotein. The lines *ey-tTA* and 205 described in this study show the highest levels of *tTA* among the lines generated to date in the laboratory and we have reproducibly obtained repression of gene activity in embryos by feeding their mothers with tetracycline in a range from 1 to 1000 μg/ml tetracycline, and in larvae, with as little as 0.1 μg/ml tetracycline. The use of tetracycline is especially appropriate to keep the inducible gene promoter silent during embryogenesis in order to direct its expression during larval development. Tetracycline concentrations ranging from 0.1 to 10 μg/ml ensure reactivation of the tetracycline-responsive promoter within 24 hours after transfer of the larvae to normal medium. It is important to point out that the amount of tetracycline required to inactivate *tTA* is both low and non-toxic. This is essential to keep the promoter inactive up to a desired stage and to ensure its fast activation upon removal from tetracycline exposure. We have found that the addition of tetracycline to the larval food does not give any toxic effect in a range of 0 to 100 μg/ml, although the development is slowed down at concentrations above 1 μg/ml. As shown with the *ey-tTA* strain, tetracycline can be used to control the timing of induction at distinct phases of development in order to define a phenocritical period. Temporal control of gene expression should also be effective during pupal development as a function of the concentration of tetracycline provided to the larvae before pupation. They can be well synchronised during this developmental period and go through a number of well-characterised stages (Ashburner, 1989). In combination with the use of the *lacZ* reporter, these features should help in determining the time course of induction of any gene driven by a *tTA*-expressing line of interest.

Our attempts to use the reverse tetracycline-controlled transactivator (*rtTA*, Gossen et al., 1995) have been unsuccessful in *Drosophila*. This transactivator is based on a mutagenized version of *tTA*, which binds the *tetO* sequences only in the presence of specific tetracycline derivatives. It corresponds to a 4-amino-acid exchange in *tetR*, which is thought to alter the conformation of the repressor and allows its binding to DNA upon association with certain tetracycline compounds (Gossen et al., 1995). Since it was originally isolated in a genetic screen in bacteria and tested successfully in mammalian cells (Gossen et al., 1995) and in transgenic mice (Kistner et al., 1996), *rtTA* might need a temperature close to 37°C to be stable. In contrast, *tTA* shows a potent activity in *Drosophila* and its negative regulation by tetracycline is not a major difficulty, as described above. Furthermore, both the repression and the kinetics of gene induction might be

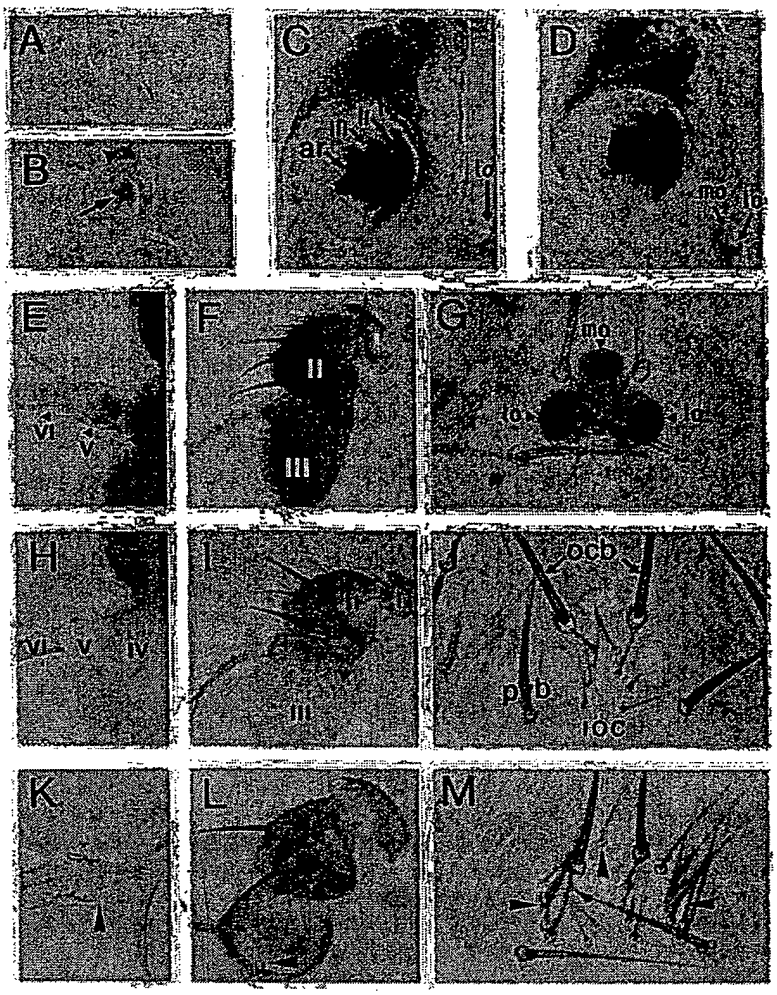

Fig. 5. Adult phenotype resulting from the directed expression of *Antennapedia* by the 205 strain. (A-D) Expression pattern of the 205 strain in the eye-antennal disc visualized by X-gal detection of β-galactosidase activity encoded by *tetO-lacZ* reporter. Discs are oriented with anterior up and dorsal left. (A) Early third instar. (B) Mid third instar; expression starts in the centralmost region of the antennal disc (arrow) and the presumptive palp region (arrowhead). (C) Late third instar larvae: expression has expanded in a wedge-shaped sector overlapping the most proximal region of the arista (ar) and the three major antennal segments (roman numerals). Expression in the lateral occellus (lo) that is not detectable in active wandering larvae is also indicated. (D) White prepupae: β-gal activity is detectable in the medium occellus (mo). (E-G) Expression of the *lacZ* reporter in the respective adult structures: the most proximal segments of the arista (E), the three antennal segments (F) and the occelli (G). (H-J) Normal phenotype of 205/+; *tetO-Antp*/+ adults raised continuously with 10 μg/ml tetracycline. Occipital, post vertical and interoccellar bristles are indicated by ocb, pvb and ioc, respectively. (K-M) Altered phenotype of 205/+, *tetO-Antp*/+ adults derived from larvae shifted from 10 μg/ml tetracycline to standard food during the late third instar. Note the thickening of the proximal segments of the arista (K, arrowhead), a bunch of new bristles on the third antennal segment and a modification in the number, the localization and the shape of the characteristic bristles of the second antennal segment (L, arrowheads). We could not determine the origin of these bristles on the basis of their morphology. Although different from the usual antennal bristles, they are not of a leg type because of the lack of the characteristic bract. The other main feature is a bunch of thick and long bristles of unknown origin close to the occelli (M, arrowheads).

increased further with one of the numerous tetracycline derivatives available. Some of them have been shown to be more potent effectors on *tTA* than tetracycline itself (Gossen and Bujard, 1993; Chrast-Balz and Hooft van Huijsduijnen, 1996).

Binary systems for controlling gene expression

The interest in using a binary system that combines an effector molecule for controlling activity of a responder promoter has been largely demonstrated with the GAL4/UAS system (Brand and Perrimon, 1993). Our method makes use of a similar experimental strategy, which allows the stable integration of any kind of construct in a parental fly strain in the absence of the transactivator. Tissue-specific activation of the gene construct is achieved in the offspring of a cross with a driver line chosen for its pattern of expression of the transactivator. The *tet* system provides a more versatile tool, owing to the possibility of controlling the timing of gene expression during development. In addition, the use of the vectors RHT and WTP facilitates the generation of driver strains expressing *tTA* under the control of previously isolated tissue-specific enhancers, and responder strains carrying any gene of interest under the regulation of the *tetO*-containing promoter. We also plan to generate a collection of *tTA*-expressing strains following the

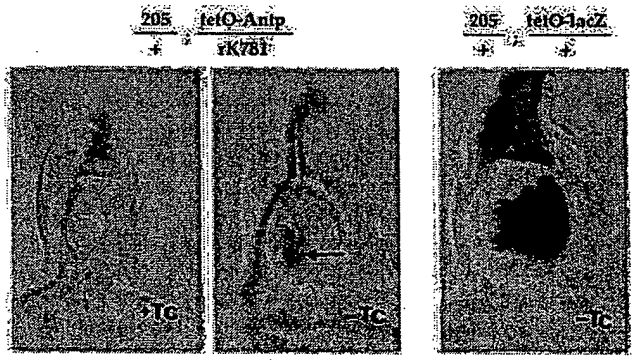

Fig. 6. Tetracycline-controlled expression of *Antennapedia* by the 205 strain activates rK781. Shown are β-galactosidase-stained antennal discs isolated from larvae raised continuously on 10 μg/ml tetracycline (+Tc) or shifted to standard food 48-72 hours before dissection (–Tc). The arrow points to the activation of rK781 in the centralmost region of the wedge-type sector of *tTA* expression, visualized on the right by the *tetO-lacZ* reporter. Eggs were collected over 12 hours from tetracycline-treated females of the genotype Cy[A405. M2]/205; rK781/rK781 mated with +/+ ; *tetO-Antp*/TM6,Tb males. Larvae of the genotype 205/+ ; *tetO-Antp*/rK781 were identified by their Tb^+ phenotype and the absence of the staining pattern due to the Cy[A405. M2] chromosome.

random integration into the genome of an enhancer detector construct with *tTA* as a reporter gene. The availability of strains expressing *tTA* in a wide variety of patterns will ensure a large number of applications for the *tet* system.

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In vivo analysis of scaffold-associated regions in *Drosophila*: a synthetic high-affinity SAR binding protein suppresses position effect variegation

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Scaffold-associated regions (SARs) were studied in *Drosophila melanogaster* by expressing a synthetic, high-affinity SAR-binding protein called MATH (multi-AT-hook), which consists of reiterated AT-hook peptide motifs; each motif is known to recognize a wide variety of short AT-rich sequences. MATH proteins were expressed specifically in the larval eye imaginal discs by means of the tetracycline-regulated transactivation system and tested for their effect on position effect variegation (PEV). MATH20, a highly potent SAR ligand consisting of 20 AT-hooks, was found to suppress *white*$^{mottled\ 4}$ variegation. This suppression required MATH20 expression at an early larval developmental stage. Our data suggest an involvement of the high AT-rich SARs in higher order chromatin structure and gene expression.

Keywords: chromatin structure/*Drosophila*/PEV/SAR

Introduction

Scaffold-associated regions (SARs), also called matrix attachment regions or MARs, are operationally defined as DNA sequences that specifically associate with the nuclear scaffold or matrix and possibly define the bases of chromatin loops (Mirkovitch *et al.*, 1984; Cockerill and Garrard, 1986; Gasser and Laemmli, 1986). SARs are very AT-rich regions of several hundred base pairs in length, that are possibly best described as being composed of numerous, irregularly spaced A tracts (short AT-rich sequences containing homopolymeric runs) (reviewed in Laemmli *et al.*, 1992). Proteins that specifically bind to SARs do not appear to interact with a precise base sequence but rather recognize certain structural features of non-B DNA, such as narrow minor grooves, DNA bends and a propensity to unwind (Bode *et al.*, 1992; Käs *et al.*, 1993).

SARs play roles in both chromosome condensation and gene expression. A recent report identified SARs as *cis*-elements of chromosome dynamics (Strick and Laemmli, 1995), while numerous publications demonstrated that SARs, in a flanking position, can strongly stimulate the expression of various heterologous reporter genes in different biological systems (Laemmli *et al.*, 1992). The SAR-mediated stimulation of transgene expression is not observed in transient assays, but only after stable integration of the test constructs into the genome (Klehr *et al.*, 1991; Poljak *et al.*, 1994). Hence, these *cis*-acting elements may exert their effect via chromatin structure, since transiently transfected DNAs are known to be poorly organized into nucleosomes.

A recent model attempts to explain the general stimulatory effect of SARs on transcription by proposing that SARs facilitate the displacement of histone H1 (a process referred to as chromatin opening) through mutually exclusive interactions with proteins of similar DNA-binding specificity, such as the high mobility group protein HMG-I/Y (Käs *et al.*, 1993). HMG-I/Y contains three short DNA-binding domains, called AT-hooks, that bind the minor groove of A tracts similarly to the peptide antibiotic distamycin (Reeves and Nissen, 1990). Histone H1, HMG-I/Y and distamycin all bind selectively to the A tracts of SARs, and competition experiments between them have demonstrated that HMG-I/Y and distamycin are 'dominant': i.e. both can displace pre-bound histone H1 from an SAR template (Zhao *et al.*, 1993). The observation that distamycin, added to cells, markedly stimulated cleavage at SARs by topoisomerase II in internucleosomal linker DNA but not at hypersensitive sites, lends *in vivo* support to this model; increased accessibility for cleavage presumably arises from the displacement of proteins, possibly histone H1 (Käs *et al.*, 1993). The model is also consistent with elegant studies demonstrating that SARs are necessary to spread open chromatin from enhancers to gene promoters (Kirillov *et al.*, 1996; Jenuwein *et al.*, 1997).

To study the role of SARs in chromosome condensation, synthetic multi-AT-hook proteins (MATH), consisting of numerous reiterated AT-hook peptide motifs derived from HMG-I/Y (Strick and Laemmli, 1995), were synthesized. Since the AT-rich SARs have enough adjacent binding sites to accommodate all the multiple, covalently linked AT-hooks, these HMG-I/Y derivatives bind SARs (both as DNA and chromatin) with exquisite specificity (Strick and Laemmli, 1995). Their effects on chromosome assembly were tested in *Xenopus* egg extracts capable of converting added nuclei to mitotic chromosomes *in vitro*. Remarkably, adding low levels of MATH20, a protein containing 20 hooks, inhibited the normal events of chromosome condensation, suggesting that SARs are *cis*-elements of mitotic chromosome dynamics (Strick and Laemmli, 1995).

To address the importance of SARs in the fruit fly *Drosophila melanogaster*, we expressed MATH20 in the larval eye imaginal disc to test for an effect on position effect variegation (PEV). We found that regulated expression of MATH20 led to a suppression of PEV, suggesting

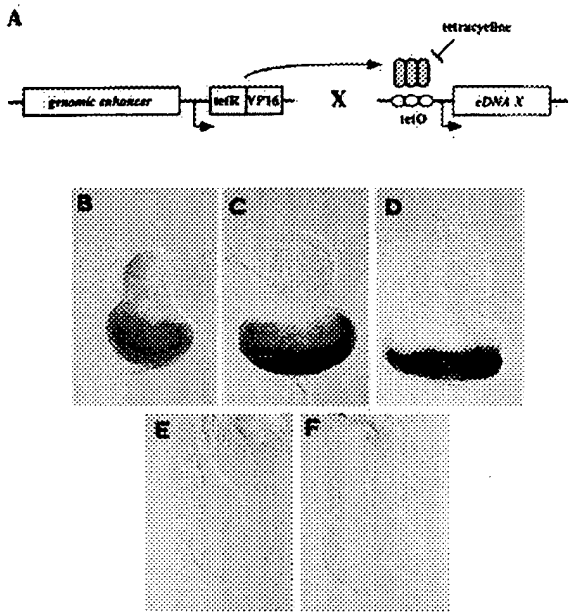

Fig. 1. Protein overexpression by means of the tetracycline-regulated transactivation system in *Drosophila*. (**A**) Schematic representation of the Tet system applied to *Drosophila* (see text for details). In (B–F), β-galactosidase staining of eye-antennae imaginal discs from early (**B** and **E**), mid (**C**) and late third instar larvae (**D** and **F**), showing the activation of a tetO–LacZ reporter construct by the *eyeless* enhancer-tTA strain. In (E) and (F), larvae were maintained on medium containing tet 0.2 μg/ml, resulting in a complete inhibition of the tTA-induced LacZ expression.

an involvement of SARs or SAR-like AT-rich regions in long-range chromatin structure and gene regulation.

Results

Targeted expression of synthetic SAR-binding proteins in Drosophila

With the aim of improving our understanding of SAR function(s) *in vivo*, we have overexpressed MATH20, the synthetic SAR-binding protein, in *Drosophila* by means of the tetracycline (tet)-regulated transactivation system (Tet system) (Bello *et al.*, 1998). Our strategy consisted of targeting expression of these proteins specifically to the eye imaginal discs during larval development and scoring for effects on *white* variegation. The principle of the binary Tet system is depicted in Figure 1A. A fly strain expressing a tet-regulated transactivating protein [tTA, a fusion of the tet repressor DNA-binding domain and the VP16 transcriptional activation domain (Gossen and Bujard, 1992)] under the control of regulatory sequences [in our case, an eye-specific enhancer identified in the intronic sequences of the *eyeless* gene (Quiring *et al.*, 1994)] is crossed to a strain containing the coding sequence for the gene of interest downstream of the tet operator sequences. The expression of this gene is then obtained in the progeny, in the same pattern as tTA is expressed, and can be tightly controlled by tet (Bello *et al.*, 1998). As shown in Figure 1B–D, by monitoring *tetO–LacZ* reporter gene expression, the *ey*-tTA strain can be used to target protein expression in the third instar eye imaginal discs, primarily in the undifferentiated eye cells (Figure 1B), and progressively only in the differentiated cells posterior to the morphogenetic furrow (Figure 1C and D). Additionally, β-galactosidase activity is also observed in the eye imaginal discs during the first and second instars (data not shown). Tet, when added to the food at a concentration as low as 0.2 μg/ml, is able to completely silence the expression of the *LacZ* reporter gene, both in early (Figure 1E) and late third instar (Figure 1F).

MATH20 suppresses PEV

In *Drosophila*, PEV refers to the mosaic expression of a gene when chromosome rearrangements place it close to heterochromatin. This heterochromatin-mediated gene silencing is proposed to be a heritable, epigenetic event that involves no alteration in the DNA content. Silenced genes are believed to be packaged into a higher order chromatin structure, or alternatively might be localized to a special nuclear compartment that confers transcriptional repression (reviewed in Karpen, 1994; Elgin, 1996). A classical example of PEV is the *white*-mottled (w^{m4}) inversion, in which the *white* gene necessary for the red pigmentation of the eye is juxtaposed close to heterochromatin of the X chromosome.

The variegating phenotype of *white*-mottled is seen as numerous clones of red, wild-type cells in an otherwise *white* mutant background (Figure 2A). Since MATH proteins are very efficient in SAR binding, including satellite III found in the centromeric heterochromatin of the X chromosome (Strick and Laemmli, 1995, and below), we reasoned that overexpressing MATH proteins specifically in the eye imaginal discs of the developing larvae might modify *white* variegation, a process known to involve chromatin structure. Females of the *ey*-tTA strain in a w^{m4} background were crossed to males of the various independent tetO-MATH20 strains. After eclosion, males of the desired genotypes (which are heterozygous for both *ey*-tTA and tetO-MATH20 transgenes, and hemizygous for w^{m4}) were kept for 5 days at 25°C and photographed. As a control, we used a line containing an empty tetO vector, showing a typical 'salt and pepper'-like mosaic expression of the *white* gene (Figure 2A). Expressing MATH20 resulted in a significant derepression of the *white* gene, and a general loss of the variegating phenotype. This suppression of PEV was clearly visible in various independent MATH20 lines (Figure 2B–F), and was shown to be highly reproducible. Quantitative analysis of the red eye pigment levels for seven independent MATH20-expressing strains is shown in Figure 3A, with PEV suppressor ratios ranging from 1.6 to 2.8 when compared with the control line (two independent control lines gave identical results). In contrast, MATH11, a less potent SAR DNA-binding protein (Strick and Laemmli, 1995), revealed no PEV-modifying effects in four independent strains (Figure 3A, dashed bars).

To test for the specificity of the MATH20-induced suppression of PEV, we made use of tet to silence the expression of the MATH20 transgene. Progeny of a cross between w^{m4}, *ey*-tTA females and tetO-MATH20-19 or empty tetO were grown on either normal or tet-containing food, under exactly the same conditions of temperature and population density as before. While tet treatment has no effect on the *white* mosaic expression in the control

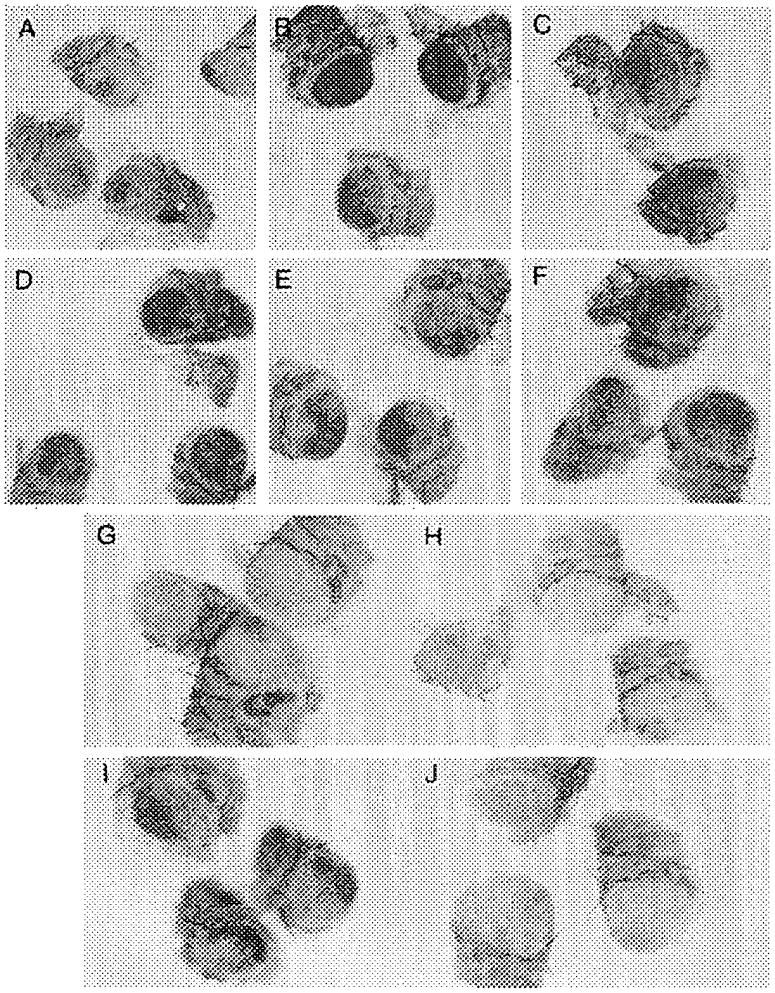

Fig. 2. Eye-specific expression of MATH20 suppresses *white* variegation. (A–F) Females of the *ey*-tTA strain in a w^{m4h} background were crossed to males of the various tetO-MATH20 strains. Progeny was grown on standard medium at 25°C. After adult eclosion, males of the desired genotype were kept for 5 days at 25°C before photography. Shown are photomicrographs of male heads of the following genotypes: (**A**) w^{m4h}/Y, control tetO/+, *ey*-tTA/+; (**B**, **E** and **F**) w^{m4h}/Y, tetO-MATH20/+, *ey*-tTA/+, with respectively strains 20-14, 20-2 and 20-19. (**C** and **D**) w^{m4h}/Y, +/+, *ey*-tTA/tetO-MATH20, with respectively strains 20-4 and 20-6. (G–J) Inhibition of MATH20-induced PEV suppressor effect by tet treatment. Females of the *ey*-tTA strain in a w^{m4h} background were crossed to males of the control tetO strain (**G** and **H**) or the tetO-MATH20-19 strain (**I** and **J**). Progeny was grown at 25°C, either on standard medium (G and I) or medium containing tet 0.2 μg/ml (H and J).

line (Figure 2G–H), it clearly inhibits MATH20-induced suppression of PEV (compare Figure 2I and J). Quantitative analysis is given for three MATH20 strains (Figure 3B). Results are shown as the ratio of OD_{480} MATH20 to OD_{480} control: while modification of *white* variegation is observed in the absence of tet (Figure 3B, open bars), tet treatment, by maintaining silent the MATH20 transgene expression, leads to eye pigment levels very similar to those of the control (Figure 3B, black bars).

We next made use of tet to examine whether suppression of PEV by MATH20 might require expression during an early developmental stage. For this purpose, 50 females of the w^{m4h}, *ey*-tTA strain were crossed to males of the control or tetO-MATH20-12 strain. One hour egg collections were made, and kept on either normal or tet-containing food. Larvae were then transferred at regular intervals to normal food. In these conditions, the transgene is kept efficiently silent, but it is activated after shifting larvae off tet following a lag period of ~12 h (Bello *et al.*, 1998). As shown in Figure 3C, PEV suppression is still observed when larvae are exposed to tet up to 60 h after egg laying (AEL). If the gene is kept silenced longer (72–120 h), then no PEV suppression is observed. The *eyeless* enhancer activity and hence MATH20 expression can be detected throughout the larval stages and up through early pupal stages when differentiated eye cells develop from precursor cells. Since activation of the transgene, during the third instar period (72 h AEL), no longer reduced PEV, we conclude that MATH20 expression in the undifferentiated cells is required to achieve suppression of PEV.

MATH20 suppresses cleavage by topoisomerase II in satellite III heterochromatin of chromosome X

Is suppression of PEV by MATH20 mediated by interactions at SARs? The w^{m4} inversion juxtaposes the *white* gene to the heterochromatin of the X chromosome. Intriguingly, the predominant component of the

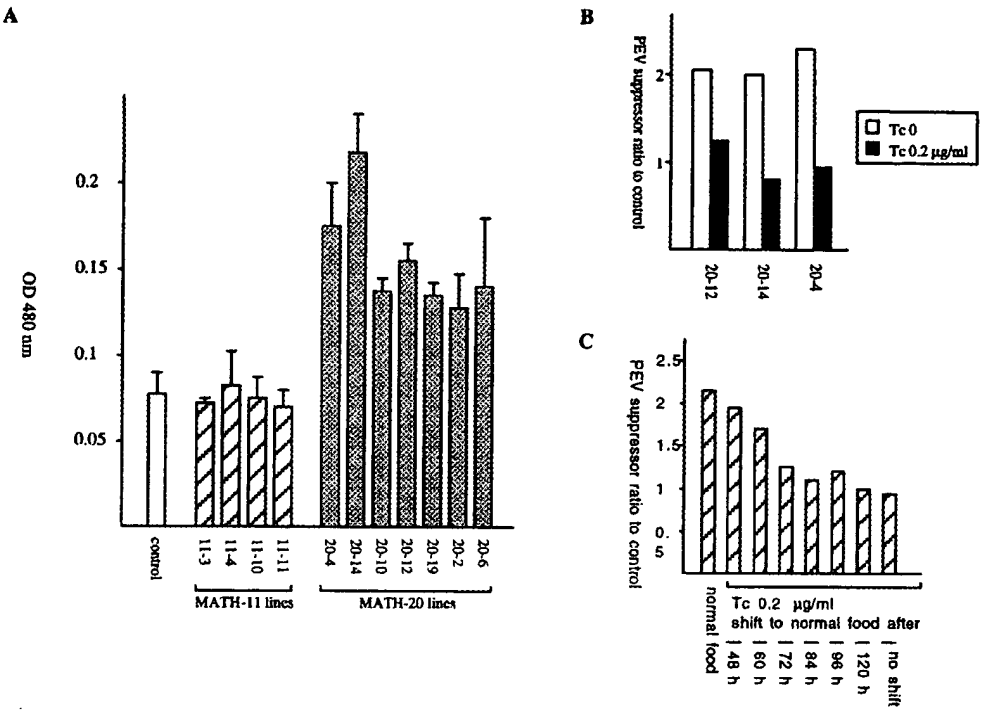

Fig. 3. Quantitation of MATH20-induced PEV suppression. (**A**) MATH20, but not MATH11, suppresses w^{m4} variegation. Females of the *ey*-tTA strain in a w^{m4h} background were crossed to males of the control tetO strain or the various tetO-MATH11 (dashed bars) and tetO-MATH20 strains (shaded bars). Quantitation of the red eye pigment levels was done on groups of 40 male heads 5 days after eclosion, and repeated 4–6 times. Standard deviations are shown as thin lines above the histograms. (**B**) Inhibition of MATH20-induced PEV suppression by tet. Females of the *ey*-tTA strain in a w^{m4h} background were crossed to males of the control tetO strain or three independent tetO-MATH20 strains. Progeny were grown at 25°C on either normal food (open bars) or food containing tet 0.2 µg/ml (black bars). Values are given as the ratio of the $OD_{480\ nm}$ of the tetO-MATH20 lines to the $OD_{480\ nm}$ of the empty tetO vector control line, and represent the average of three independent measurements. (**C**) Females of the *ey*-tTA strain in a w^{m4h} background were crossed to males of the control tetO strain or tetO-MATH20-12 strain. Progeny were kept on normal or tet-containing food (0.2 µg/ml). Larvae were transferred to normal food at the indicated times after egg laying. The red eye pigment levels were measured in groups of 30 male heads, and repeated three times. Values are given as the average ratio of the $OD_{480\ nm}$ of the tetO-MATH20-12 strain to the $OD_{480\ nm}$ of the control strain.

heterochromatin is satellite III, also called the 1.688 or the 359 bp repeat satellite (Hsieh and Brutlag, 1979). Two or three repeats (718–1017 bp) of this satellite behave as fully fledged SARs *in vitro*; they preferentially bind nuclear scaffolds, topoisomerase II and HMG-I/Y (Käs and Laemmli, 1992; Karpen, 1994). Thus, the inverted *white* gene appears juxtaposed to a giant, reiterated SAR of ~11 Mb.

Topoisomerase II is one of the growing number of proteins associated with heterochromatin (Rattner *et al.*, 1996). Although it is not known whether topoisomerase II is implicated in heterochromatin formation, it is one of the few proteins for which the site of interaction can be studied by stabilizing the so-called cleavage intermediate using cytotoxic drugs. Thus, this protein serves here as a convenient, heterochromatin-associated reporter protein which is expected to monitor the interaction of MATH20 in this chromatin. Moreover, topoisomerase II is known to be specifically enriched over satellite III heterochromatin, as revealed by microinjection of fluorescent topoisomerase II into *Drosophila* embryos (Denburg *et al.*, 1996). This preferential interaction was also borne out by previous studies that demonstrated a major topoisomerase II cleavage site once per satellite III repeat; this 359 bp repeat contains two positioned nucleosomes, and the major topoisomerase II cleavage site occurs in one of two nucleosomal linker regions as depicted in Figure 4B.

Does MATH20 interfere with topoisomerase II cleavage in satellite III? We addressed this question using *Xenopus* egg extracts. These extracts are known to carry out faithfully many cellular processes. Indeed, we noted that the endogenous topoisomerase II of such extracts generated a cleavage ladder in satellite III repeats of *Drosophila* Kc nuclei that is indistinguishable from the one observed in cells (Figure 4A, lanes 1 and 2). Interestingly, this cleavage ladder is suppressed specifically in a dose-dependent manner by MATH20 (lanes 2–5). In contrast, no inhibition is observed by added HMG-I/Y (lane 6) which binds much more dispersively to the genome. In conclusion, MATH20 can interfere specifically with topoisomerase II cleavage in satellite III, strongly suggesting that the MATH20 protein encoded by the transgene expressed in flies is very likely to bind specifically the heterochromatin of chromosome X.

Discussion

The variegated phenotype of *white* mottled flies is the result of a large inversion in the X chromosome that places the *white* gene adjacent to centomeric heterochromatin. PEV is due to a stochastic inactivation of the *white* gene in some but not other cells at an early stage of eye development, followed by clonal maintenance through later stages. The resulting pattern of *white* gene expression

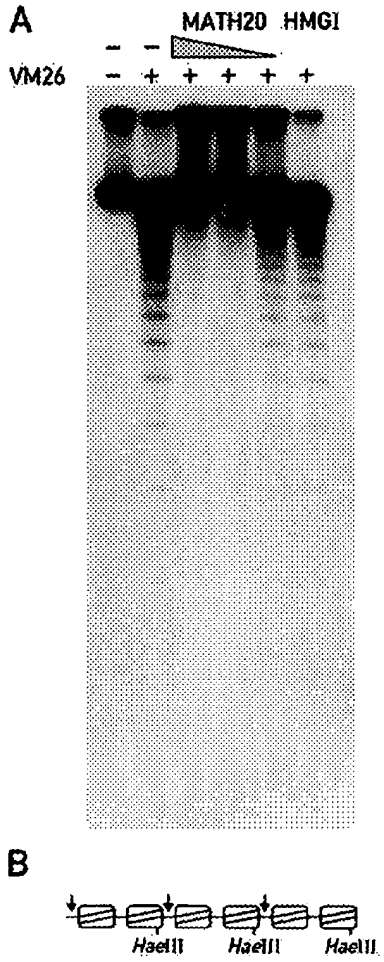

Fig. 4. Specific suppression by MATH20 of topoisomerase II cleavage in satellite III of chromosome X. This figure demonstrates that MATH20 specifically inhibits topoisomerase II cleavage in satellite III, which is the predominant component of the heterochromatin of chromosome X. (A) Isolated Kc *Drosophila* nuclei were incubated in mitotic *Xenopus* egg extracts in the presence of different concentrations of MATH20 or HMG-I/Y. The topoisomerase II cleavage activity subsequently was monitored in satellite III by treating the extracts for 10 min with VM26. The DNA samples were displayed on a 1.2% agarose gel and the Southern blot hybridized with a satellite III repeat probe. VM26 and proteins were added as indicated at the top. The sample in lane 1 received no VM26 and no MATH20, and that in lane 2 received VM26 only. Samples in lanes 3–5 contained 80, 40 and 20 ng of MATH20, respectively. The sample in lane 6 contained 80 ng of HMG-I/Y. (B) The repeat structure of satellite III chromatin, which consists of two nucleosomes per 359 bp repeat unit. Topoisomerase II cleaves (arrow) once per unit, in every other nucleosomal linker region (Käs *et al.*, 1993).

is observed in the eye as patches of pigmentation. Morphologically, PEV is observed on polytene chromosomes as a spreading of heterochromatic structures into euchromatic genes (Elgin, 1996). Current evidence favors a model according to which silencing proteins of the centromeric heterochromatin spread into the juxtaposed euchromatic region by a cooperative assembly process. This spreading may be a consequence of the inversion by removing putative boundary elements that otherwise delimit heterochromatin (Locke *et al.*, 1988).

We have shown here that specific expression of the artificial high-affinity SAR-binding protein MATH20 in the developing eye imaginal discs results in suppression of *white* variegation. Using the tetracycline gene regulation system, we demonstrated that suppression of PEV requires the expression of MATH20 in the undifferentiated eye cells and is observed if this transgene is activated up to 60 h after egg laying. In contrast, no suppression is observed upon activation of MATH20 during the late third instar period. Interestingly, suppression of PEV by MATH20 was also observed for the *Brown*^{D} variegating rearrangement (data not shown). We found no effect on *white* variegation by expression of MATH11; this protein with only 11 AT-hooks has a 7-fold lower binding affinity (K_D = 18.2 pM) for SARs than MATH20 (2.6 pM). Consequently, proportionally higher amounts of protein were required to affect chromosome condensation in *Xenopus* extracts (Strick and Laemmli, 1995). Hence, it might be necessary to express MATH11 in the eye imaginal disc with a stronger promoter to achieve an effect on *white* variegation. The differential effect of MATH11 versus MATH20 underscores the notion that suppression of PEV is a specific phenomenon; it appears to be related to the binding strength of the effector. Adding MATH to a mitotic *Xenopus* extract led to the formation of abortive condensation products (Strick and Laemmli, 1995), and microinjection of MATH20 (but not HMG-I/Y) blocked HeLa cells in late G_2-phase following passage through S-phase (R.Strick, R.Peperkok and U.K.Laemmli, in preparation). In agreement with this, we have observed that higher levels of embryonic and larval expression of either MATH20 or MATH11 led to lethality. Thus, suppression of PEV required tissue-specific expression of MATH20 at a low level that does not interfere with cell division.

The inverted *white* gene is juxtaposed to a giant 11 Mb reiterated SAR in the form of satellite III repeats. MATH20 binds there with great specificity (Strick and Laemmli, 1995) and is able to interfere with the activity of topoisomerase II (Figure 4). This observation establishes proof of the principle that MATH20 can interact with or displace a pre-bound protein associated with heterochromatin. Although we cannot rule out other possible models, it is tempting to explain the effect of SAR on gene expression, chromatin opening and PEV by extending a model put forward by Laemmli and colleagues (Laemmli *et al.*, 1992). In this model, certain proteins (called compacting proteins here) interact with SARs or certain AT-rich satellites cooperatively; this can lead to either chromatin folding, chromosome condensation (looping) or formation of heterochromatin. Conversely, other proteins such as HMG-I/Y and their monster derivatives, MATH, bind non-cooperatively to SARs and can displace the compacting proteins by disrupting their cooperative interactions, hence resulting in chromatin unpacking. These alternative chromatin states are governed by the binding strength and the relative local level of the chromatin packaging proteins versus those that undo it. Of importance for these considerations is the extent of SAR repetition at a given region. Since an assembly of cooperatively interacting proteins becomes energetically more favorable with increasing repeats, certain packaging proteins are expected to polymerize preferentially onto satellite III chromatin over individual SARs. In contrast, the non-cooperative MATH

proteins would bind dispersively to single and reiterated SARs. Thus in a cell, while a single SAR in euchromatin may promote chromatin opening and stimulation of gene expression (Jenuwein *et al.*, 1997), a reiterated SAR could result in silencing.

It is easy to explain the effect of MATH20 on PEV by simple considerations based on the above model. The high affinity of MATH20 for SARs could allow it to bind to the satellite III region or other AT-rich regions, thus disrupting the cooperative interaction of the compacting proteins. This in turn would energetically disfavor the spreading of the polymerizing proteins into the flanking euchromatic region. As the probability of inactivating an adjacent euchromatic gene diminishes, one expects to observe suppression of PEV. It is impossible at present to obtain direct evidence for the model, and for a direct *in vivo* proof for MATH20 binding to satellite III. PEV appears to be a complex, poorly understood process, and the nature of the *cis*-DNA elements involved in heterochromatin formation has yet to be elucidated. The results reported here could also provide a clue about the nature of these *cis*-acting elements involved in PEV; the data suggest that this chromatin state might be mediated in part by proteins that interact with AT-rich repeats, such as those of satellite III.

Materials and methods

Fly strains

The yw^{67c23} strain was used as recipient for injections. P-element-mediated germ line transformation was done using standard procedures (Spradling and Rubin, 1982a,b). For each construct, multiple independent lines were established, and the chromosomal location of the inserted transgene was determined by standard genetic analysis using balancer chromosomes. The $white^{mottled\ 4}$ inversion, $In(1)w^{m4b}$ strain, was used to score the effects of MATH proteins on *white* variegation. PEV-modifying effects were quantified by red eye pigment measurement (Ashburner, 1989), on groups of 40 male heads of the desired genotype, kept for 5 days at 25°C after eclosion. Flies were grown at 25°C on standard medium, unless specified otherwise in the text. Tetracycline (Sigma) was used at 0.2 µg/ml as described (Bello *et al.*, 1998). Fly strains expressing tet-VP16 transactivator are described elsewhere (Bello *et al.*, 1998).

Plasmid constructs

Further details of the cloning procedures are available upon request. MATH11 and MATH20 cDNAs (Strick and Laemmli, 1995) were inserted into pWTP (marked with *miniwhite*) (Bello *et al.*, 1998) and/or pYTP (marked with *yellow*). pYTP was constructed by inserting a tet operator–P promoter–SV40 polyadenylation signal cassette from pWTP into pY vector, made by inserting the *yellow* gene from the YES vector (Patton *et al.*, 1992) into Carnegie 4 (Rubin and Spradling, 1983).

β-Galactosidase activity

β-Gal stainings were done as described (Ashburner, 1989), on glutaraldehyde-fixed eye-antennal imaginal discs from early and late third instar larvae.

Topoisomerase II cleavage assay

Kc nuclei were isolated (Mirkovitch *et al.*, 1984) and added to *Xenopus* egg extracts (Strick and Laemmli, 1995) to a final concentration of 10 000 nuclei/µl of extract, incubated at 21°C for 1 h and subsequently treated with 50 µM VM26 for 10 min. The reactions were stopped and isolated DNA samples were electrophoresed in a 1.2% agarose gel, electroblotted to nylon membranes and hybridized to satellite III repeat probes as described (Käs *et al.*, 1993).

Acknowledgements

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A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program

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We have developed a tetracycline-repressible female-specific lethal genetic system in the vinegar fly *Drosophila melanogaster*. One component of the system is the tetracycline-controlled transactivator gene under the control of the fat body and female-specific transcription enhancer from the yolk protein 1 gene. The other component consists of the proapoptotic gene *hid* under the control of a tetracycline-responsive element. Males and females of a strain carrying both components are viable on medium supplemented with tetracycline, but only males survive on normal medium. A strain with such properties would be ideal for a sterile-insect release program, which is most effective when only males are released in the field.

There is increasing interest in biological methods for control of insect pests, in part because of increasing resistance to chemical insecticides and other factors such as environmental effects of insecticides. A biological method that has proven to be effective in the field for the area-wide control of some insects is called the sterile insect technique (SIT; ref. 1). SIT involves raising large numbers of insects that are then sterilized before field release. If sufficient numbers of competitive insects are released, most of the wild females in the field mate with the released sterile males and thus produce no viable offspring (1, 2). SIT can result in suppression or eradication of the target insect (1, 2). Successful past SIT programs include the eradication of screwworm from North America (2), tsetse fly from Zanzibar (3), Queensland fruit fly from Western Australia (4), and melon fly from the Okinawa islands (5). SIT has also been used for eradication or suppression of the Mediterranean fruit fly (medfly) in various parts of the world (1, 6).

For medfly, SIT has been shown to be most effective when only sterile males are released in the field (7). Current medfly SIT programs use so-called "genetic sexing strains" that facilitate the large-scale separation of males from females (8). The strains are made by classical genetic methods involving the isolation of Y:autosome translocations, where the translocation carries a dominant wild-type allele for a selectable gene (9). For example, genetic sexing strains have been made that are homozygous for a recessive temperature-sensitive lethal allele on chromosome 5, and males carry a Y:5 translocation that includes a wild-type allele of the temperature-sensitive lethal gene (9). In these genetic sexing strains, only male embryos survive incubation at the nonpermissive temperature. However, the strains can breakdown under mass rearing conditions because of male recombination (8, 9). An alternative method of making a genetic sexing strain is to use genetic engineering (10). Transgenic insects are made by using transposable elements that seem to have a broad host range (10). For example, transgenic medflies have been made by using the *Minos* (11) and *piggyBac* (12) transposable elements. Similarly, *piggyBac* has been used to make transgenic silk moth (13). Further, transgenic mosquitoes (*Aedes aegypti*) have been made by using the *Hermes* (14) and *mariner* (15) transposable elements.

Our aim was to construct a "terminator" gene that, under certain conditions, is lethal to transgenic female flies but otherwise has no effect on either male or female viability. Herein, we report the development of such a system with the vinegar fly *Drosophila melanogaster*. The terminator gene we choose was the proapoptotic gene *head involution defective* (*hid*; ref. 16), because ectopic expression of *hid* can lead to organismal death caused by induction of apoptosis (16). *hid* expression was regulated by the tetracycline-controlled transactivator (tTA), which is inactive in the presence of tetracycline (17). Expression of tTA was controlled with the female-specific enhancer from the *Drosophila* yolk protein 1 (*yp1*) gene (18). Because the components of the system are either conserved (yolk protein genes; ref. 19) or known to function in both *Drosophila* and mammalian cells (*hid*, ref. 20; tTA, refs. 17 and 21), we believe the system could be used to make genetic-sexing strains for a variety of insect pests that can be genetically engineered.

Methods

Construction of *yp1-tTA* and *tetO-hid*. To construct *yp1-tTA*, a 158-bp DNA fragment containing the female-specific transcription enhancer of the *yp1* gene (18) was obtained by PCR with *D. melanogaster* DNA as template. The forward primer was 5′-ATC TAT ATT TTA TGC ATT TAT TTG ATC-3′, and the reverse primer was 5′-AAT AGA CAC GGG GCC TAC CTA T-3′. The 50-μl reactions contained 200 ng of genomic DNA, 200 nM forward and reverse primers, 200 μM dNTPs, 1.6 mM $MgCl_2$, and 1 unit of eLONGase (Life Technologies, Grand Island, NY) in buffer supplied by the manufacturer. Reactions were heated to 94°C for 3 min then cycled 35 times (30 s at 94°C; 30 s at 47°C; 30 s at 68°C) in a Perkin–Elmer 9600 thermocycler. A product of the correct size was purified by agarose gel electrophoresis, digested with *Eco0109I* then incubated at 75°C for 10 min to inactivate the enzyme. The DNA was then treated for 15 min at 25°C with the Klenow fragment of DNA polymerase I (New England Biolabs) in buffer supplied by the manufacturer supplemented with 33 mM dNTPs. After incubation at 75°C for 10 min to inactivate the enzyme, the DNA was digested with *BclI*. The resulting 124-bp fragment was inserted into the *Bam*HI and *Eco*RV sites of the pBluescript II KS (−)vector (Stratagene). Cloning of the correct fragment was confirmed by DNA sequencing. The fragment containing the *yp1* enhancer was excised with *Not*I and *Asp718* and inserted into the *Not*I and *Asp718* sites of the tTA transformation vector W.H.T. (21). W.H.T. is a CaspeR-derived vector with the *Not*I and *Asp718* sites immedi-

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Abbreviations: tTA, tetracycline-controlled transactivator; SIT, sterile insect technique; medfly, Mediterranean fruit fly.

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ately upstream of the *hsp70* minimal promoter that is used to drive expression of the tTA coding sequence.

To construct *tetO-hid*, a 3.9-kilobase *Eco*RI fragment containing the complete *hid* ORF (16) was inserted into the *Eco*RI site of the *tetO* vector W.T.P.2 (21). W.T.P.2 is also a CaspeR-derived vector that contains seven copies of *tetO*, a minimal promoter, and a unique *Eco*RI site between the *hsp70* leader and *hsp70* poly(A) region.

***Drosophila* Stocks.** Flies were usually raised on medium that had a high yeast content but contained no added corn meal (100 g of active dry yeast, 100 g of sugar, and 16 g of agar per liter). Alternative medium compositions that were used are described in the text. Crosses were performed at 25°C. All stocks not specifically mentioned have been described by Lindsley and Zimm (22). For germ-line transformation, constructs were coinjected into *y w* embryos with the Δ2,3 helper plasmid (23) by using the standard procedure (24). Single F_1 progeny displaying a nonwhite eye color were backcrossed to *y w* then bred to homozygosity. Linkage of P [w^+] was determined by following w^+ segregation in the appropriate crosses.

Recombinant lines carrying both *yp1-tTA* and *tetO-hid* constructs were selected by first crossing homozygous *yp1-tTA* and *tetO-hid* lines where both lines had insertions on the third chromosome. The virgin female offspring were collected then mated with *w*; *Tb/TM3, Sb* males, and 100 male offspring from this cross were mated singly with *w*; *Tb/TM3, Sb* females on normal medium and also on medium supplemented with tetracycline (10 μg/ml). Crosses raised on normal medium that lacked w^+ females were identified as probable recombinants. Homozygous lines were established by crossing w^+ non-*Sb* males and females. Dissected larvae, pupae, and adults were stained for β-galactosidase activity by using the method of Simon and Lis (25).

Results

The Tetracycline-Controlled Female-Killing System. The system was designed such that female flies would die in the absence of tetracycline because of widespread cell death in the fat body. The system is shown schematically in Fig. 1. Expression of tTA is controlled by the female- and fat-body-specific enhancer from the *yp1* gene (18). Binding of tTA to tetO results in activation of expression of the proapoptotic gene *hid*. Induction of apoptosis in fat body results in female-specific lethality, because the fat body is an important tissue for metabolism and food storage in insects. Females are viable when raised on culture medium supplemented with tetracycline, because the antibiotic inhibits the binding of tTA to tetO.

To test the system, homozygous tetO-*hid* and *yp1-tTA* lines were crossed, and the offspring were raised on either normal medium or medium supplemented with tetracycline (10 μg/ml; Table 1). Thus, the offspring of the crosses carry one copy of each construct. We found that, for most of the crosses raised on normal medium, there was a highly significant decrease in female viability. In particular, for the cross *yp1-tTA* line 19 with *tetO-hid* line 53, 99.7% of the offspring were male. Female lethality occurred during the pupal stage. From the crosses where most of the females died (e.g., *yp1-tTA* line 19 crossed with *tetO-hid* line 27), the females that emerged either died shortly after eclosion or were sterile and showed a variety of defects such as wing bubbles. We attribute the variable level of female killing to the position of integration affecting the level of expression of either the *tTA* or *hid* genes. In contrast, for crosses raised on medium supplemented with tetracycline, we found that males and females were equally viable.

For an SIT program that typically involves raising millions of flies, it would not be practical to mate separate lines each carrying one of the components of the female-killing system.

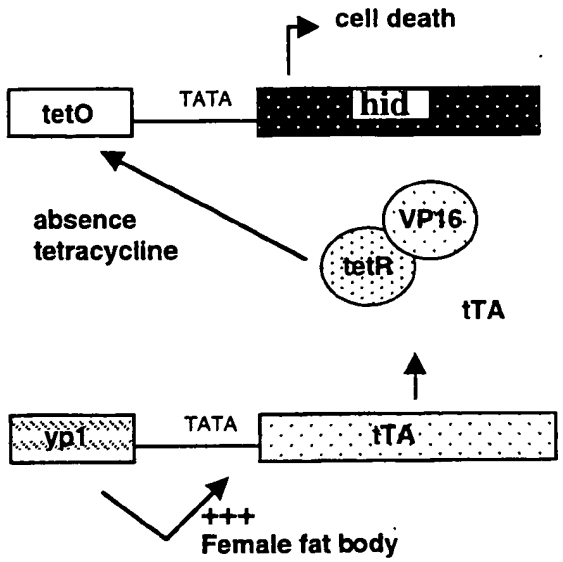

Fig. 1. The tetracycline-regulated female-killing system. Expression of tTA is controlled with the female- and fat-body-specific transcription enhancer from the *yp1* gene (18). In the absence of tetracycline, tTA binds to tetO and induces expression of the proapoptotic gene *hid*. The loss of fat body results in female-specific lethality. In the presence of tetracycline, females are fully viable, because the binding of tTA to tetO is inhibited, switching off *hid* expression.

Therefore, we wanted to determine whether a line could be maintained that carried both components of the system. *yp1-tTA* line 19 was mated with *tetO-hid* line 53; recombinant offspring were identified and either bred to homozygosity or maintained with a balancer chromosome. Only males survived when the homozygous line (which carries two copies of each construct) was raised on normal medium, but both males and females survive equally when raised on medium supplemented with tetracycline (Table 2). Further, the homozygous males from the culture raised without tetracycline are fertile. Thus, we conclude that it is possible to maintain a line that contains both components of the female-killing system.

Female- and Fat-Body-Specific Expression of tTA. To confirm that in *yp1-tTA* lines tTA was expressed in the female fat body, *yp1-tTA* line 19 was crossed with a line carrying a *tetO-lacZ* reporter gene (21). The offspring of the cross were dissected and stained for

Table 1. Viability of males and females carrying one copy each of the *tetO-hid* and *yp1-tTA* constructs

yp1-tTA line	*tetO-hid* line	Tetracycline, 10 μg/ml	No. female	No. male	Percentage male
19	53	−	1	330	99.7
19	53	+	376	362	49.1
19	27	−	18	195	91.5
19	27	+	175	138	44.1
19	8	−	61	99	61.9
19	8	+	46	33	41.8
6	53	−	2	89	97.8
6	53	+	181	162	47.2
22	53	−	216	194	47.3
22	53	+	209	189	47.5
30	53	−	47	120	71.8
30	53	+	165	112	40.4

Table 2. Tetracycline-repressible female-specific lethality of a recombinant line with two copies of the *tetO-hid* and *yp1-tTA* constructs

Tetracycline, 10 μg/ml	Male	Female
−	222	0
+	139	186

The recombinant line was obtained by mating *tetO-hid* line 53 with *yp1-tTA* line 19 and selecting for recombinant offspring.

β-galactosidase activity. We found strong β-galactosidase expression in the fat body of female larvae (Fig. 2), pupae, and adults (data not shown) raised on normal medium but not in females raised on medium that contained tetracycline (Fig. 2). There was little staining in the fat body of male larvae (Fig. 2), pupae, or adults (not shown) raised on either normal medium or medium supplemented with tetracycline (Fig. 2).

A Yeast-Rich Diet Is Essential for Induction of Female Lethality. Female *D. melanogaster* that are starved from eclosion show a basal level of yolk protein synthesis that is rapidly induced by supplying a normal diet (26). The control element for this diet-dependent response was mapped to an 890-bp fragment upstream of the *yp1* gene (26). Further studies showed that the nutritional response could be mediated by any of several smaller fragments of the 890-bp fragment, including the 124-bp enhancer used in this study (27). In the experiments described above, the flies were raised on a relatively rich medium that contained 100 g of active dried yeast per liter. Because the cost of the culture medium can be significant in a SIT program (1), we wanted to determine whether the female-killing system was affected by diet. *yp1-tTA* line 19 was mated with *tetO-hid* line 53 and raised

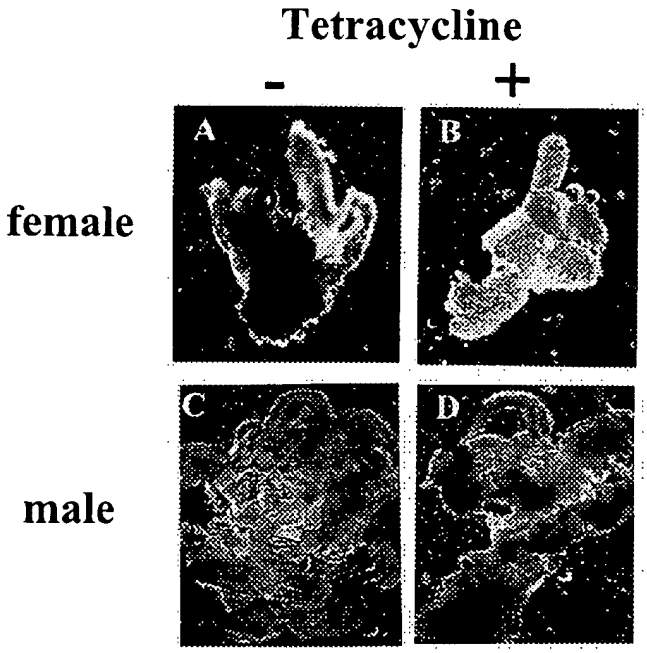

Fig. 2. Expression of tTA is confined to the female fat body and is inhibited by tetracycline. Climbing third instar larvae were sexed, dissected, and stained for β-galactosidase expression (25). Strong staining was seen in fat body of female larvae raised on normal medium (*A*) but not medium that contained tetracycline (10 μg/ml; *B*). Little staining above background level was seen in fat body from male larvae raised on either normal medium (*C*) or medium supplemented with tetracycline (*D*).

Table 3. Medium with a high yeast content is required for efficient induction of female-specific lethality

Medium type*	Yeast, g/liter	Cornmeal, g/liter	No. male†	No. female†
High yeast	100	0	330	1
Intermediate yeast	62	0	103	1
Low yeast	32	0	201	205
Intermediate yeast + cornmeal	62	107	235	5
Low yeast + cornmeal	32	107	170	226

*All types of culture medium contained 100 g of sugar and 16 g of agar per liter.

†Males and females are the offspring of a cross between *yp1-tTA* line 19 with *tetO-hid* line 53 and thus carry one copy of each construct.

on medium that contained a low, intermediate, or high amount of yeast. We found that the efficiency of the female-killing system increased with the level of yeast in the diet (Table 3). Addition of corn meal to low-yeast medium did not affect female viability. Thus, efficient induction of female lethality depends on diet, particularly the level of yeast in the culture medium.

Discussion

We have developed a repressible female-specific lethal system that under certain conditions results in complete female lethality. Further, we have maintained a strain homozygous for both components of the system for several generations on medium supplemented with tetracycline. When transferred to medium without tetracycline, the males that emerge are viable and fertile. Such properties are suitable for a strain that is to be used in a sterile release program. Ideally, it would be preferable if female-specific lethality occurred at the embryonic stage rather than pupal stage, because of the costs associated with raising large numbers of larvae. However, such a system would require a female-specific promoter or enhancer that is expressed earlier in development than the yolk protein genes. Although the system has been developed to make a strain suitable for a sterile release program, it may also be possible to release fertile males to control the target insect, because female viability depends on tetracycline in the diet. From the matings between the released males and females in the field, only male offspring will survive, and these males in turn will produce only male offspring. However, the results presented in this study suggest that the efficiency of this approach could depend on the quality of the diet of the insects in the field. A relatively poor diet may result in survival of some female offspring of the released males, unless the terminator gene is very effective.

The amount of induced ectopic cell death is very sensitive to the level of ectopic *hid* expression (28), which in the female-lethal system depends directly on the level of tTA expression. Transgene expression is influenced by the local chromatin environment, and tTA expression is controlled by the *yp1* enhancer, which may explain why the efficiency of the system depends on the sites of integration of the constructs and the level of yeast in the diet. The position effects could be minimized by bracketing the *yp1-tTA* and *tetO-hid* constructs with insulator elements (29). The effect of diet on female lethality is consistent with previous studies that showed that the *yp1* fat body enhancer is responsive to diet, particularly yeast (26, 27). It will be of interest to determine whether the diet response is mediated via either the sex-specific double-sex protein or the proteins that bind to the b-zip or w3 sites of the enhancer, because the binding sites for all three proteins are required for enhancer function *in vivo* (30). Genes involved in the diet response potentially could be identified by carrying out sensitive genetic screens (31) for mutations that either enhance female lethality on a low-yeast diet or suppress lethality on a high-yeast diet. Such screens

potentially could also identify genes that act downstream of *hid* in the induction of apoptosis in fat body. The efficiency of the system could potentially be improved by including a second proapoptotic gene such as *reaper* or *grim* also controlled by a tetracycline-responsive element. In the central nervous system, midline cells *reaper*, *grim*, and *hid* seem to act cooperatively to induce apoptosis (32, 33). Further, *reaper* and *grim* but not *hid* seem to activate specifically the *Drosophila* caspase DCP-1 *in vivo* (34).

Although we have demonstrated that the system is effective in *Drosophila*, we think it is likely that the system will be applicable to other insects. The tTA is functional in both *Drosophila* (21) and in mammalian cells (17) and is thus likely to be functional in other insects. Similarly, the *Drosophila hid* gene has been shown to induce apoptosis in mammalian cells (20). However, it is possible that the *Drosophila yp1* enhancer may not retain the correct tissue and sex specificity in other insects. Indeed, the regulatory regions from the housefly yolk protein genes show the correct tissue specificity but not sex specificity in *Drosophila* (35), suggesting that it might be necessary to isolate the yolk protein genes from the insect species of interest. Yolk protein genes have been isolated from a number of insect species including the medfly (36). The availability of these genes, methods for germline transformation (11, 12), and the current use of SIT to control the medfly make this species attractive for testing the repressible female-lethal genetic system. Our results suggest that culture medium will be an important consideration in developing this system in other insects.

After submission of this article, a similar system for controlling female viability was reported by Thomas *et al.* (37). In their system, the female- and fat-body-specific enhancer from the yolk protein 3 (*yp3*) gene (38) was used to drive expression of tTA. The terminator gene regulated by tTA is *Ras64B*^{val12}, which encodes a constitutively active Ras, a key component of the receptor tyrosine kinase signaling pathway (39). Thomas *et al.* (37) report 100% lethality for females carrying one copy of each of the *yp3-tTA* and *tetO-Ras64B*^{val12} constructs when raised on normal food that lacks tetracycline (37). It is difficult to compare the efficiency of the two female-killing systems directly. Both *yp3-tTA* lines tested by Thomas *et al.* (37) were equally effective, which may indicate that the *Ras64B*^{val12} gene is a more effective terminator than the *hid* gene. Additionally, the *yp3* enhancer may be stronger or less sensitive to position effects than the *yp1* enhancer used in this study. However, the two *yp3-tTA* lines tested by Thomas *et al.* (37) were chosen on the basis of strong expression of the *white*$^{+}$ marker gene (D. D. Thomas and L. S. Alphey, personal communication), and thus, the *yp3-tTA* construct may have integrated into sites that were favorable for high levels of tTA expression. Further, the medium used contained high levels of yeast (D. D. Thomas and L. S. Alphey, personal communication). Like the *yp1* enhancer, the *yp3* enhancer is also responsive to diet (40). It will be desirable to compare the two female-killing systems directly by crossing a *yp1-tTA* line with a *tetO-Ras64B*^{val12} terminator line and also crossing a *yp3-tTA* line with a *tetO-hid* line on normal medium that contains either low or high yeast.

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Appendix D

A transgene-based, embryo-specific lethality system for insect pest management

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Biological approaches to insect pest management offer alternatives to pesticidal control. In area-wide control programs that cover entire regions, the sterile insect technique (SIT) can be used to successfully suppress economically important pest species by the mass release of sterilized pest organisms. However, conventional sterilization by ionizing radiation reduces insect fitness, which can result in reduced competitiveness of the sterilized insects. Here we report a transgene-based, dominant embryonic lethality system that allows for generation of large quantities of competitive but sterile insects without the need of irradiation. The system involves the ectopic expression of a hyperactive pro-apoptotic gene that causes embryo-specific lethality when driven by the tetracycline-controlled transactivator (*tTA*) under the regulation of a cellularization gene enhancer-promoter. We have successfully tested this system in *Drosophila melanogaster*. The embryonic lethality can be suppressed maternally, which will allow it to be combined with transgenic female-specific lethality systems to raise only vigorous but sterile males.

Many insects heavily damage crops and forests or transmit deadly diseases to animals and humans. Current control efforts mostly rely on the use of insecticides, but chemical control can have adverse consequences and the costs of developing new chemical products to circumvent insecticide resistance are increasing. In genetic control based on SIT, mass-reared, sterile insects are released into the field, resulting in infertile matings and thereby reducing the pest population[1,2]. In SIT programs the terms 'sterility' or 'sterile insect' do not usually indicate that the individuals generate no sperm or eggs, but rather refer to the transmission of dominant lethal mutations that kill the progeny[1,2]. Because of its species specificity, SIT is considered an ecologically safe procedure and has been successfully used in area-wide approaches to suppress or eradicate in entire regions pest insects such as the pink bollworm *Pectinophora gossypiella* in California[3], the tsetse fly *Glossina austeni* in Zanzibar[4], the New World screwworm *Cochliomyia hominivorax* in North and Central America[5], and various tephritid fruit fly species in different parts of several continents[6].

For the Mediterranean fruit fly (medfly) *Ceratitis capitata*, male-only releases increase effectiveness of the SIT[7]. Separation of undesirable females has been based on genetic sexing strains[8]. However, recent advances in insect transgenesis[9–11] have promoted the development of transgene-based methods for sex separation that are based on the female-specific expression of a conditional dominant lethal gene. Such systems have been examined in the model insect *D. melanogaster* and might be transferable to other insect pest species[12,13].

Knipling stated in 1955 that in addition to the requirement for mass rearing and sexing, SIT also necessitates that "sterilization methods must produce sterility without serious adverse effects on the mating behavior or length of life of the males" and that "if females of a species mate more frequently, the sperms from sterile males must be produced in essentially the same number and compete with sperms from fertile males"[1]. Conventional sterilization is based on ionizing radiation that causes chromosome fragmentation. Chromosome fragments without centromeres will not be transmitted correctly to the progeny. However, radiation also has adverse effects on viability and sperm quality, which results in reduced overall competitiveness of the sterilized individuals[14–17]. Certain insects with holocentric chromosomes, such as many lepidopteran pest species, do not possess chromosomes with defined centromeres. Instead the whole chromosome has centromeric properties, which allow fragmented chromosomes to be inherited correctly. Therefore, sterilization of these insect pests requires very high doses of radiation, which often greatly impair fitness[18]. Thus large quantities of sterilized insects are required to inundate the pest population, which results in high operational costs.

Here we describe a transgenic approach to cause sterility without interfering with the adult phase of the insect life cycle or with gametogenesis. The sterility is based on the transmission of a transgene combination that causes dominant embryo-specific lethality in the progeny. This allows for the generation of vigorous and potent sterile insects, with males being able to transfer competitive sperm. For the effector gene, which will cause organismal lethality, we chose the pro-apoptotic gene *head involution defective* (*hid*) (also known as *Wrinkled* (*W*)). This gene induces cell death when expressed ectopically[19]. To avoid downregulation of HID by Ras signaling pathways, we used the phosphoacceptor-site mutant allele hid^{Ala5} (ref. 20). To limit the effect of the transgenes to the embryonic stage, we used enhancer-promoters of genes that are expressed at high levels but are specific to the blastoderm stage. In *D. melanogaster*, the genes *serendipity α* (*sryα*) and *nullo* encode structural components of the microfilament network that are specifically required for blastoderm cellularization[21,22]. To establish conditional embryonic lethality, we used a suppressible binary expression system based on tTA (ref. 23).

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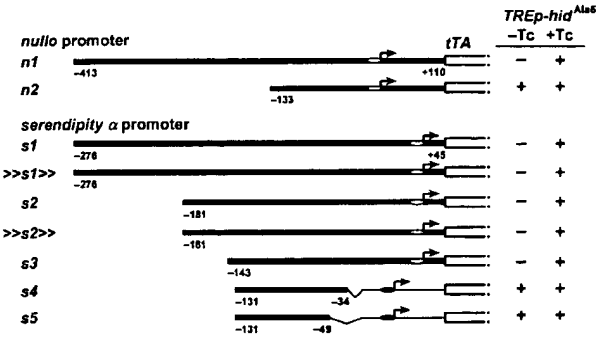

Figure 1. Schematic representation of *nullo-tTA* and *sryα-tTA* fusion constructs, including functional analysis. Left, >> indicates tandem repeats of the 5′ HS4 chicken β-globin domain insulator sequence. With the exception of *s4* and *s5*, gene fusions are at the ATG start codon. The *tTA* coding region is represented as an open box. Numbers refer to the *nullo* or *sryα* transcriptional initiation site. *s4* contains the enhancer element described as sufficient for blastoderm-specific expression[27] fused to the minimal promoter from the *hsp70* gene. Right, tetracycline-suppressible lethality after cross to >>*TREp-hid*^{Ala5}>> line M1.II (nomenclature is as described in Table 1). Emerging from tetracycline-free (–Tc) or tetracycline-containing medium (+Tc, 100 μg/ml), F1 progeny were analyzed to identify those carrying both transgenes as indicated by eye-specific fluorescence of both the enhanced yellow fluorescent protein (indicating *tTA* constructs) and enhanced cyan fluorescent protein (indicating *TREp-hid*^{Ala5} constructs)[30]. For lethal (–) combinations, double fluorescence was not observed among a total of 59–185 F1 individuals, whereas nonlethal (+) combinations showed double fluorescence in 18–29%, which corresponds to the expected Mendelian ratio of 25% (both parents heterozygous).

We have functionally examined this system in *D. melanogaster* and can demonstrate that *hid*^{Ala5} causes embryonic lethality when driven by *tTA* under the control of the enhancer-promoter from a cellularization gene. The lethality is specific to embryonic stages and can be suppressed by tetracycline provided maternally to the egg. Strains homozygous for the transgene combination can be propagated only on tetracycline. Males from these *D. melanogaster* strains are competitive in laboratory mating assays and transmit the transgene combination that causes dominant embryonic lethality in offspring at high efficiency. The transgene-based suppressible embryo-specific lethality system enables the production of competitive sterile insects without irradiation and will therefore be of interest to improve conventional SIT and widen its applicability.

Results

A suppressible, dominant, embryo-specific lethality system. To restrict the detrimental effects of a dominant lethality system to the embryo, we looked for *cis*-regulatory elements that are active during the earliest possible stages of embryogenesis and whose activity is entirely confined to these stages. The *D. melanogaster* cellularization genes *sryα* and *nullo* are expressed specifically at the blastoderm stage[24,25]. Their strong and ubiquitous, but stage-specific, expression seemed suitable to drive an embryo-specific lethality system. To protect driver and effector constructs from undesirable insertion site–specific enhancer effects that might cause additional cell lethality and therefore weaken the competitiveness of adult males, we flanked several transgene constructs with a tandem repeat of the insulator element (indicated hereafter by >>) comprising the constitutive 5′ HS4 hypersensitive site of the chicken β-globin domain[26].

The 5′ enhancer-promoter regions of *sryα* and *nullo* share sequence motifs[21], and variations in the length of the *sryα* enhancer-promoter mediate different expression levels of a heterologous reporter gene but do not significantly change the expression pattern[27]. We examined a series of *sryα* and *nullo* enhancer-promoter *tTA*-fusion transgenes (Fig. 1), and determined whether they could drive sufficient *tTA* expression to cause lethality through the tTA-response element (*TRE*)-controlled effector gene *hid*^{Ala5} (*TREp-hid*^{Ala5}). For each driver transgene, three independent insertions were crossed against the same >>*TREp-hid*^{Ala5}>> effector line and their progeny were analyzed for survival of flies carrying both transgenes in the presence and absence of tetracycline. With the exception of driver transgenes *n2*, *s4*, and *s5*, all other constructs caused lethality that could be suppressed by the addition of tetracycline (Fig. 1). The lack of lethality with constructs *s4* and *s5* is probably due to the reduced activity of the heterologous promoter (Fig. 1) in these constructs[27].

Homozygous embryonic lethality strains. To ensure successful application, all progeny must inherit the dominant embryonic lethality system, which requires that the released insects carry both driver and effector constructs in homozygous condition. By crossing and recombining several insulated and non-insulated effector (>>*TREp-hid*^{Ala5}>>; *TREp-hid*^{Ala5}) and driver (*n1*; *s1*; >>*s1*>>; *s2*; >>*s2*>>) lines, 48 combinations were set up. In ten cases both constructs could be bred to homozygosity (Table 1) on food containing tetracycline. For all ten strains, withdrawal of tetracycline resulted in a lack of progeny, indicating that homozygosity for the transgene combinations (two copies each) causes lethality in the absence of tetracycline (Table 1, column 4).

Nonetheless, to apply the embryonic lethality system, it was necessary that the heterozygous transgene combination suffice to cause lethality, as homozygous males or females will mate with their wild counterparts and the resulting progeny will inherit only one copy of each transgene. Of the ten strains, four produced progeny when males were crossed to wild-type virgin females (Table 1, column 5). This indicated that not all driver and effector combinations are dominantly lethal—that is, mediate sufficiently strong activation of *hid*^{Ala5} to cause lethality when present in one copy. The insulated *TREp-hid*^{Ala5} effector seems to cause lethality more easily than the

Table 1. Characterization of homozygous embryonic lethality strains (EL#)

tTA	*TREp-hid*^{Ala5}	EL#	Progeny		Suppression of sterility[a]	
Construct, line[b]	Line[b]		Homozygous[c]	Heterozygous[d]	+Tc (μg/ml)	+Tc → –Tc (μg/ml)
n1, M4.II	>>F1.II>>	45	–	–	100	100
>>*s1*>>, M3.II	M3.III	29	–	–	100	100
>>*s1*>>, M3.II	>>F1.II>>	43	–	–	100	100
>>*s2*>>, M7.III	>>F1.II>>	13	–	–	10	30–100
>>*s2*>>, M5.II	>>M5.II>>	41	–	–	3	10–30
>>*s2*>>, M8.II	**>>M5.II>>**	**42**	–	–	**3**	**10**
>>*s2*>>, M7.III	M2.II	20	–	+	100	100
>>*s2*>>, M6.III	M3.III	46	–	+	1–3	3–10
>>*s2*>>, M7.III	M3.III	47	–	+	3	10
s2, M3.II	M3.III	31	–	+	3	30

[a]Minimal tetracycline concentration required to produce progeny on diet with the indicated concentration (+Tc) or after transfer to tetracycline-free diet from diet containing the indicated concentration (+Tc → –Tc).
[b]In our strain nomenclature, .II indicates location on second and .III on third chromosome. M, male founder individual; F, female founder individual.
[c]Progeny (+) or no progeny (–) on tetracycline-free diet after pre-incubation of strains on tetracycline-free diet for 4 d.
[d]Single males (20 independent matings each) were analyzed for progeny after crossing to wild-type virgins.
The boldfaced strain EL#42 was used for further analyses.

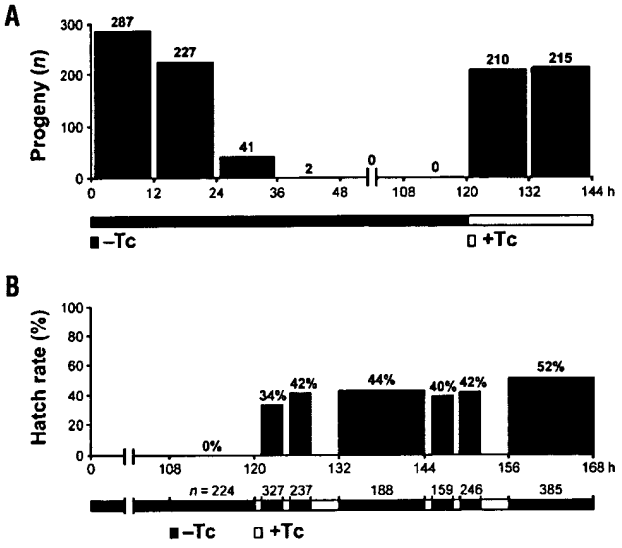

Figure 2. Reversibility of strain EL#42 phenotype. (A) Strain EL#42 flies were transferred from tetracycline-containing (100 µg/ml) to tetracycline-free medium (–Tc, black bar). After 120 h, tetracycline-containing yeast (100 µg/ml) were added to the medium (+Tc, white bar). Progeny of 12 h egg lay intervals were monitored (embryos were collected, raised, and emerging adults were scored; absolute numbers of adult flies are given above the columns). (B) Maternal suppressibility of embryonic lethality. Flies were incubated on tetracycline-free medium (–Tc) for 5 d. To deplete dietary protein, flies were kept during the 72–120 h interval on apple juice plates without yeast. Lack of progeny was confirmed for the time interval 108–120 h. At 120 h, the indicated feeding scheme (black-white bar) was applied: 1 time unit (1 h or 4 h) on apple juice plates with tetracycline-containing yeast (+Tc; 100 µg/ml) followed by 3 time units (3 h or 12 h) on tetracycline-free apple juice plates. The larval hatch rate (hatched larvae per total number of collected eggs) was determined for the tetracycline-free intervals (total number of eggs given above feeding scheme bar).

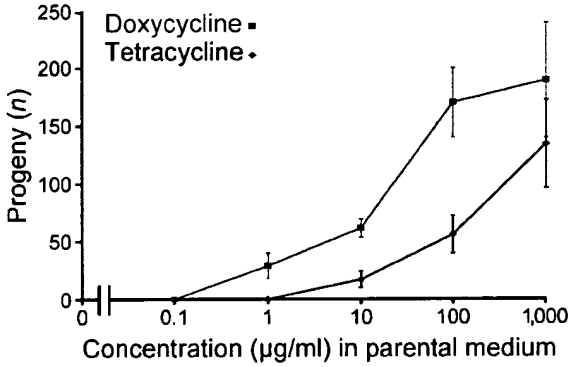

Figure 3. Maternal suppression of strain EL#42 sterility by the tTA inhibitors doxycycline (light gray) and tetracycline (black). Equal numbers of parental flies (15 male and 15 female), reared on medium containing inhibitor at the concentration indicated, were transferred to inhibitor-free medium and assessed for number of adult progeny. Data given for concentration values above 0.1 µg/ml are the means ± s.d. of seven to nine independent experiments.

non-insulated version, which probably reflects higher levels of *hid*^{Ala5} expression. Only those combinations of driver and effector that mediate close to 100% lethality when heterozygous can be deployed.

To identify the minimal tetracycline concentration needed to propagate the strains, flies were bred on different tetracycline concentrations (0.1, 1, 3, 10, 30, 100, and 1,000 µg/ml). All strains could be kept at 100 µg/ml tetracycline, but several strains could even be raised on 3 µg/ml tetracycline (Table 1, column 6). As expected, for most strains without dominant embryonic lethality in a heterozygous situation, lethality is also suppressed by low amounts of tetracycline (Table 1, compare columns 5 and 6). The amount of tetracycline needed to suppress lethality probably correlates with the level of *hid*^{Ala5} expression. Only strain EL#20 is exceptional in this respect, as high levels of tetracycline are required to suppress lethality in homozygous condition, whereas the heterozygous condition is viable. This could be due to a transvection effect on the particular effector insertion M2.II, which may cause the homozygous condition to be significantly better expressed than the heterozygous condition.

In summary, of the ten homozygous strains, six demonstrated potential for use in an embryo-specific lethality system. These strains contain various driver and effector combinations as well as different insertions of them (Table 1). This indicated that there should be no need for particular insertions or constructs, which is important when such a system is to be transferred to an insect species that is not as easily transformable as *D. melanogaster*.

Maternal suppressibility and reversibility of embryonic lethality. In principle, the transgenic embryonic lethality system should be compatible with a transgenic female-specific lethal system that is based on the same effector construct[12]. Such a combination would allow production of competitive sterile males only, which is preferable for many SIT applications. To establish such a combined system, it will be important that embryonic lethality and female sex-specific lethality occur at distinct stages of the life cycle. Moreover, it would be best if embryonic lethality could be suppressed by tetracycline provided maternally to the egg and embryo[28]. Then the generation to be released could be grown on food lacking tetracycline. The maternal storage of tetracycline would suffice to suppress the embryonic lethality, but female-specific lethality in larval or adult stages would occur. This would result in the automatic rearing of sterile males only, without the need to separate the sexes or use irradiation to induce sterility.

By determining the parental tetracycline concentrations (0.1, 1, 3, 10, 30, 100, and 1,000 µg/ml) needed to suppress the embryonic lethality system in the next generation (Table 1, column 7), we were able to show that in all strains lethality could be suppressed by maternal contribution of tetracycline to the egg and embryo. We can actually grow these stocks efficiently for one generation after transfer onto tetracycline-free medium, which indicates that there is no major transgene-induced larval or adult lethality. However, we have not done rigorous comparisons to determine whether there are any small effects on viability. Should such small effects exist, it would be hard to clearly determine whether they are a result of simple transgene presence, insertion site problems, founder effect problems, or leaky expression.

More detailed analyses regarding the maternal suppression of lethality were done with strain EL#42, which showed suppression at concentrations as low as 10 µg/ml (Table 1, column 7). EL#42 flies were transferred from food containing tetracycline (100 µg/ml) onto tetracycline-free food. A massive decline in progeny was observed in the interval from 24 to 36 hours after tetracycline withdrawal, and no viable progeny were present after 48 hours (Fig. 2A). Interestingly, the sterility could be reversed by retransfer onto tetracycline-containing medium.

To examine whether maternal uptake rather than contact of the eggs with the new tetracycline-containing food cause reversion of the phenotype, tetracycline-withdrawn EL#42 flies were allowed to feed on tetracycline-containing yeast (100 µg/ml) for 1 hour and then allowed to lay eggs on tetracycline-free medium. Within 3 hours after the flies finished feeding on the yeast with tetracycline, lethality was strongly reduced (Fig. 2B). This shows how efficiently tetracycline in the maternal food is transferred to the eggs,

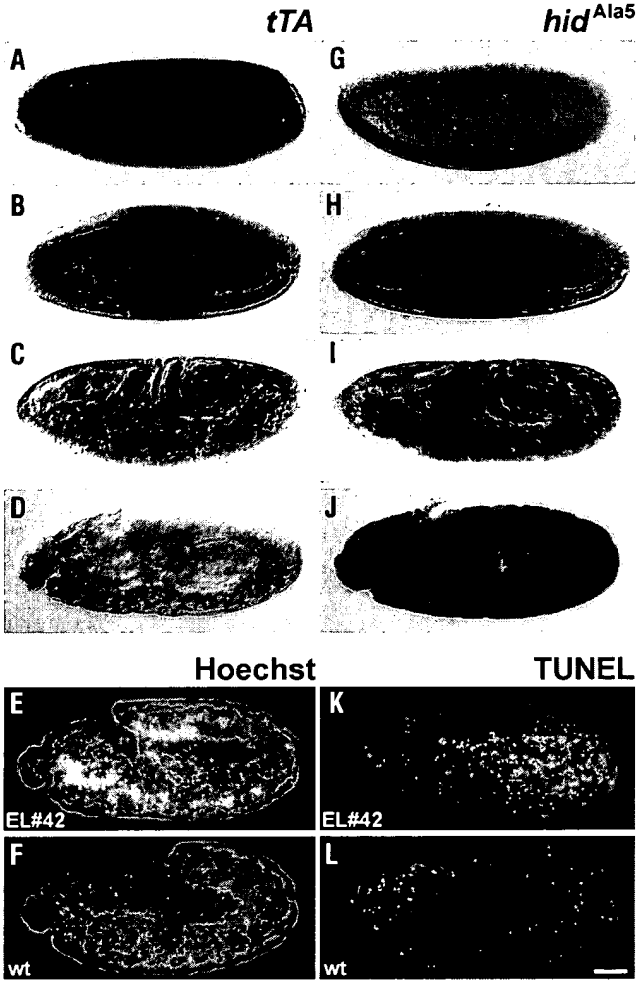

Figure 4. Embryonic lethality caused by increased cell death in embryos from wild-type virgins mated with EL#42 males. (A–D) *tTA* expression detected by *in situ* hybridization. Embryos are oriented with anterior to the left and dorsal up. The *tTA* transgene initially shows uniform mRNA distribution in the syncytial blastoderm (A). After the onset of cellularization an intense broad-banded staining is observed (not shown) which resolves at the mid-cellularization stage into four stripes (B) and vanishes soon after completion of cellularization with traces being detectable until the end of gastrulation (C). At later embryonic stages, *tTA* expression could not be detected (D). These *tTA* expression patterns are in accordance with the pattern described for wild-type *sryα* transcripts[21] and were also observed for three other independent lines of >>*s2*>>. (G–J) tTA-controlled *hid*^{Ala5} expression is absent from the syncytial blastoderm embryo (G) and is first observed at the onset of gastrulation (H). The intensity of the signal increases during germband elongation (I,J). The phenotypic consequences of tTA-induced *hid*^{Ala5} expression (E,K) were analyzed in comparison to wild-type (wt) embryos (F,L) by TUNEL (K,L) and Hoechst (E,F) staining, which indicate a failure in germband retraction and an increase in cell death. Scale bar, 50 μm (L).

leading to suppression of the embryonic lethality. Spatial separation of the tetracycline diet and medium for egg deposition also resulted in a reversion of EL#42 sterility (data not shown). This indicates that the lack of viable progeny caused by withdrawal of tetracycline is reversible and that continual production of offspring on tetracycline-free medium is in principal achievable by providing tetracycline-containing diet separately. Moreover, the reversibility of the sterility means that the transgene combination does not cause any major problems at adult stages.

Suppressibility by tetracycline and analogs. We compared tetracycline to doxycycline and anhydrotetracycline (ATc), two commonly used tetracycline analogs, for their efficiency in suppressing embryonic tTA maternally in strain EL#42. For ATc, the threshold for growth was higher than that for tetracycline: on food containing less than 1,000 μg/ml of ATc, the flies did not grow and at a concentration of 1,000 μg/ml so few progeny were obtained that comparative maternal suppressibility assays could not be carried out. In contrast, among doxycycline-fed mothers, progeny were produced at a concentration of 1 μg/ml, signifying a threshold lower by a factor of 10 than that for tetracycline (Fig. 3).

Mechanism of embryo-specific lethality. We have demonstrated that the embryonic lethality strains are easily bred in sufficient numbers on inhibitor-containing food. In the following we focus on the sterility and vigor of the males obtained from strain EL#42. Because many agricultural pest species are most detrimental to the crop during their larval stage, it is very important that the transgene-mediated lethality affect the embryo and not allow larvae to hatch. To identify the stage during which the transgene combination is active and causes embryonic lethality in a situation resembling that during field application, we examined the expression profiles of the *tTA* driver (Fig. 4A–D) and the *hid*^{Ala5} effector (Fig. 4G–J) transgenes in embryos derived from a cross of EL#42 males with wild-type females.

In this situation, *tTA* expression was restricted to the blastoderm and gastrulation stages (Fig. 4A–C) and could not be detected at later stages (Fig. 4D), thereby resembling endogenous *sryα* expression[21]. Expression at later stages was also not observed in the homozygous strain EL#42 or in the homozygous line >>*s2*>> M8.II (data not shown). *hid*^{Ala5} expression was first seen at the onset of gastrulation (Fig. 4H) and became very strong during germband elongation (Fig. 4I, J). The observed time lag between transient expression of *tTA* and detectable accumulation of *hid*^{Ala5} mRNA is typical for binary expression systems, and the strong delayed expression of *hid*^{Ala5} is likely due to the stability of tTA.

Because the *hid*^{Ala5} antisense RNA probe also hybridizes to the endogenous *hid* mRNA, it is important to note that neither the homozygous >>*TREp-hid*^{Ala5}>> line M5.II nor strain EL#42 under conditions of tetracycline suppression gave rise to an expression pattern differing from the pattern observed and described for wild-type control embryos[19] (data not shown). This indicates the absence of proximal enhancer effects and the tetracycline-mediated suppression of effector transgene expression, respectively.

At the stage when germband retraction normally occurs, heterozygous EL#42 embryos started to show severe morphological defects. To examine if embryonic lethality correlates with increased levels of apoptotic cell degeneration, we carried out terminal deoxynucleotidyltransferase–mediated dUTP-biotin nick end-labeling (TUNEL). Compared to wild-type controls (Fig. 4F, L), heterozygous EL#42 embryos show strong TUNEL labeling (Fig. 4E, K), which suggests that severe cell death results from the ectopic expression of *hid*^{Ala5} and accompanies the first visible morphological abnormalities that cause embryonic lethality. In conclusion, the *tTA* and *hid*^{Ala5} expression profiles indicate that the lethality is restricted to the embryo, and the observed phenotype implies that larval hatching cannot take place.

Efficiency of embryonic lethality. To examine the efficiency of the dominant embryo-specific lethality system, we crossed single males of strain EL#42 to five virgin wild-type females. For each of three different temperatures, the hatch rate was determined in 30 independent crosses. Only sporadic larval escapers were observed, with hatch rates varying between 0.07% at 18° C (n = 18,000 eggs), 0.01% at 25° C (n = 23,000 eggs) and 0.00% at 29° C (n = 21,000 eggs). This shows that the embryo-specific lethality system is highly efficient in preventing larval hatching in the temperature range examined.

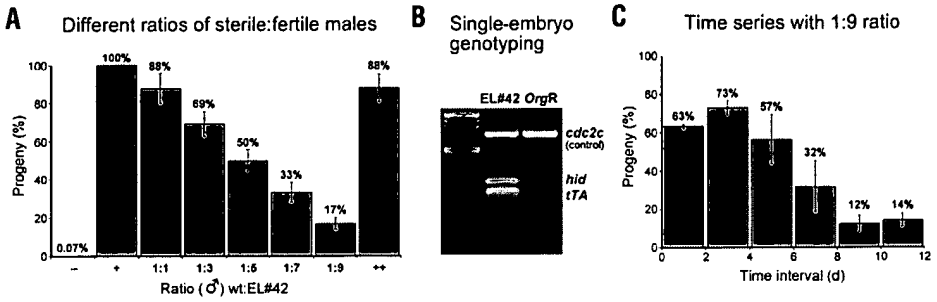

Figure 5. Competitiveness of EL#42 males. (A) Competition for virgin wild-type (wt) females: 15 virgin wild-type females and 15 wild-type males were placed together with different numbers of EL#42 males (15 EL#42 males (1:1)–135 EL#42 males (1:9)). For control matings, 15 virgin wild-type females were crossed with either 15 EL#42 males (–), 15 wild-type males (+), or 150 wild-type males (++), respectively. The flies were allowed to lay eggs for 2 d in fly bottles on tetracycline-free medium and after 13 d the number of adult progeny was recorded. Numbers are normalized to positive control (+). The mean ± s.e.m. of 14 independent experiments is indicated. (B) Multiplex-PCR to identify the genotype of single embryos in competition assays. Left lane shows as length standard the 100 bp DNA Ladder (New England Biolabs, Beverly, MA). Middle lane shows PCR fragments specific for *tTA* (191 bp) and *hid* cDNA (253 bp) whose presence indicates fertilization by EL#42-derived sperm. Both middle and right lane show a PCR fragment for the endogenous *cdc2c* gene (0.7 kb), which is used as an indicator for successful PCR reactions. The lack of the *tTA*- and *hid* cDNA-specific bands in the right lane indicates fertilization by non-EL#42 male. (C) Time course of EL#42 male competition for non-virgin females: on day 0, 135 EL#42 males were added to pre-mated *white* females and males (15 each) and a time series of six consecutive 2-d egg laying intervals was analyzed for adult progeny. Numbers are normalized to control matings without transgenic males (+). The mean ± s.e.m. of three independent time series are indicated.

Competitiveness of males transmitting embryonic lethality. To provide a marked improvement over irradiation, a transgenic approach to generating insects that transmit embryonic lethality to offspring should provide highly competitive individuals. To examine the ability of strain EL#42 males to reduce the number of progeny when competing directly with fertile wild-type males, different ratios of transgenic to wild-type males were subjected to competition for the same number of virgin wild-type females. At a nine-fold excess, transgenic males lowered numbers of progeny to an average of 17% of the expected numbers of progeny (Fig. 5A). Stress-induced lowering of progeny numbers due to the presence of ten-times the number of males is not significant (Fig. 5A, ++ control; $P = 0.105$ as determined by *t*-test), which indicates that the strong lowering of progeny numbers at the 1:9 ratio must be a consequence of effective competition by the transgenic males.

There seems to be a density-dependence effect on competitiveness. When calculating the index of mating success (ratio of the frequency of observed matings to the frequency of expected random matings)[29], EL#42 males have comparatively more mating success when applied at higher ratios and reach almost full mating success when applied at a 1:9 ratio (Fig. 5A). The reason for this effect is uncertain but might have to do with female choice or the polyandry of *D. melanogaster*.

The reduction in competitiveness of EL#42 males at lower ratios might be due to the *white* background of this strain, because white-eyed males are much less effective than wild-type males when competing for the same females[29]. To examine this at the 1:1 ratio, we allowed 50 EL#42 males to compete with either 50 *white* mutant or 50 wild-type males, respectively, for 50 wild-type virgin females. Single embryos of these crosses were analyzed by multiplex PCR to identify their fathers (Fig. 5B). When EL#42 males competed with *white* males, 28.2% ($n = 124$) of the embryos were sired by the EL#42 males, compared to only 5.6% ($n = 144$) when EL#42 males competed with wild-type males. EL#42 males are therefore five times more competitive against *white* than against wild-type males, confirming the negative effect of the *white* mutation on competitiveness. In conclusion, these competition experiments indicate that the transgenes do not have a major deleterious effect on competitiveness in this laboratory mating assay.

To simulate a situation transgenic males of a polyandrous species will face during the course of SIT, we crossed EL#42 males in nine-fold excess to pre-mated fertile *white* males and females. We observed a significant decline in the numbers of progeny after an incubation time of six days, resulting in the lowering of progeny numbers to an average of 12% of the expected number of progeny after eight days (Fig. 5C). This suggests that sperm competition can efficiently take place and shows that a clear reduction in the number of progeny is achievable by this system in a polyandrous insect species such as *D. melanogaster*.

Discussion

The results demonstrate that transgene-based, suppressible, dominant embryo-specific lethality systems can be created to generate competitive sterile insects without the need of irradiation. We tested the functionality of such systems in *D. melanogaster* and established several stable strains that are homozygous for the necessary transgene combinations. The applied transgene constructs are potentially transferable to other dipteran pest species, as the *cis*-regulatory control element of the cellularization gene *sry*α is evolutionary conserved within drosophilid species[21,27] and *tTA* is functional in *D. melanogaster*[12,13,28] as well as in mammalian cells[23]. All the constructs are based on broad-range non-autonomous *piggyBac* vectors[30] that include a widely applicable marker for transgenesis based on eye-specific expression of the green fluorescent protein[31]. Thus examination of this system should be straightforward in pest species for which germline transformation protocols have been established. In those pest species to which the transgene combinations cannot be directly applied, homologs of cellularization-specific genes and their enhancer-promoters should be identifiable, as most insect species undergo superficial cleavage patterns. Approaches that generate sterile insects without ionizing radiation will be especially valuable for lepidopteran pest species with holocentric chromosomes, because those are difficult to sterilize by irradiation without severely affecting fitness. Transgenic lepidopterans transmitting embryonic lethality to their progeny can be expected to be more competitive and as a result fewer insects will have to be released to achieve the same effect.

We tested suppressible embryonic lethality strains in *D. melanogaster* laboratory mating assays, and the transgenes did not have a major effect on competitiveness or adult viability. However, when transgenic systems are transferred to an actual insect pest species for application purposes, it will be important to investigate the detailed costs that the presence of transgenes, potential leaky expression, insertion-site problems, or founder-effect problems have on fitness, fecundity, longevity, and mating success in contained natural environments. This should enable the identification of the most effective constructs and the most suitable insertion strains. Moreover, in such experiments the abundance at which the transgenic insects will be most efficient must be determined.

The transgenic system for suppressible, dominant, embryo-specific lethality should be directly compatible with transgenic female-specific lethality systems to establish automated rearing of sterile males without the need of irradiation or gender separation. Heinrich and Scott recently described a female-specific lethal system that uses the promoter of

the yolk protein 1 gene to drive via *tTA* the pro-apoptotic effector gene *hid*[12]. Because of shared components and the similar range of tetracycline concentration needed to suppress the lethality phenotypes, the two systems could be integrated with each other. Such strains would then carry two independent *tTA* drivers (embryo-specific and female-specific) and the *hid*^{Ala5} effector. They would be kept on tetracycline-containing food and progeny would be collected on tetracycline-free medium. The maternal contribution of tetracycline would allow suppression of embryonic lethality, but the females would die at later stages when the female-specific driver was activated. Thus, only males would survive. When mated to naturally occurring females, the progeny of these males would then die as embryos. Due to the late phase of female lethality, however, females would be co-raised up to pupal stages.

The suppressible, dominant embryonic lethality system could also be combined with classic genetic sexing strains that would make it possible to kill females at early developmental stages by expressing temperature-sensitive lethal mutations[8]. This would eliminate the considerable costs of rearing females. Furthermore, when raised on food lacking tetracycline, females could also transmit the dominant embryonic lethality to their progeny. Hence the system could also be used in cases where the release of both sexes, or only females, might be more advantageous.

SIT programs should minimize the potential ecological risk of releasing transgenic organisms and might serve as appropriate systems for first evaluations of the environmental impact of transgenic insects[32]. The lack of progeny from the released insects should serve as a biological safety mechanism impeding vertical transmission of the transgenes. Therefore, the transgenes should not be able to invade wild populations and should disappear from the ecosystem with the cessation of the SIT program. In addition, a male sterility system should be suitable for initial stability tests of mass rearing in transgenic-based SIT programs, as traditional irradiation procedures could be applied additionally in case such strains become unstable.

However, before any release, measures should be taken to ensure that a transgenic program is as safe as possible. Transposons used for genomic integration of the transgenes must be non-autonomous and chosen so that no endogenous transposon activity will be present. To avoid cross-mobilization of transposons, vectors that allow effective immobilization by deletion or rearrangement of transposon ends should be generated. Moreover, introduced transgenes must not contain positively selectable drug-resistance markers. Recently developed fluorescent transformation markers[11] might be more appropriate, as they do not seem to provide advantages to carrier organisms and therefore will minimize the risk of a rare, but possible, horizontal gene transfer. In addition, these markers will allow the ratio of released sterile to wild-type pest insects to be determined, which is of key importance for monitoring a successful SIT program[33].

Experimental protocol

Insulated *piggyBac* transformation vectors. A 2.4 kb 5′ HS4 insulator tandem repeat was cloned as a *Eco*RI-*Bam*HI fragment (Klenow-blunted) from pJC13-1 (ref. 26) into *Bsi*WI-cut, Klenow-blunted pSL-3xP3-EYFPaf to yield pSL-3xP3-EYFPaf5′HS4, as well as into *Bgl*II-cut, Klenow-blunted pBac{3xP3-EYFPaf} and pBac{3xP3-ECFPaf} (ref. 30) to yield pBac{3xP3-EYFPaf5′HS4} and pBac{3xP3-ECFPaf5′HS4}, respectively. The 2.7 kb *Not*I-*Asc*I fragment from pSL-3xP3-EYFPaf5′HS4 was introduced into *Not*I- and *Asc*I-cut pBac{3xP3-EYFPaf5′HS4} and pBac{3xP3-ECFPaf5′HS4} to yield pBac{3xP3-EYF*P* > >af>>} and pBac{3xP3-ECF*P* > >af>>}, respectively. The insulator elements frame the unique *Asc*I-*Fse*I sites in 3′–5′ orientation. Plasmids with directly repeated insulator elements were amplified in STBL2™ competent cells (Life Technologies, Rockville, MD). (EYFP is enhanced yellow fluorescent protein; ECFP is enhanced cyan fluorescent protein.)

***TREp-hid*^{Ala5} effector constructs.** The *hid*^{Ala5} coding region was cloned as a 3.9 kb *Cla*I-*Bam*HI fragment from pBluescriptSK-hid^{Ala5} (provided by A. Bergmann) together with a 0.2 kb *Asp*718- and *Cla*I-cut PCR-product from pSLfa_UASp_fa (ref. 34) into *Asp*718- and *Bam*HI-cut pSLfa_TRE-SV40_fa (ref. 34) to yield pSLfa_TREp-hid^{Ala5}_fa. For PCR amplification of the 0.2 kb *P* promoter and first intron (without transposase initiator ATG)[35], we used primers CH_5′PIAsp718_2 (5′-CCGGTACCTCGATAGC-CGAAGCTTACC-3′) and CH_3′PIClaI_2 (5′-CCATCGATGGAAT-GAACAGGACCTAACGC-3′).

The final *TREp-hid*^{Ala5} effector constructs, pBac{3xP3-ECFPaf; TREp-hid^{Ala5}} and pBac{3xP3-ECFPaf; >>TREp-hid^{Ala5}>>}, were cloned by introducing the 5.0 kb *Asc*I fragment into *Asc*I-cut pBac{3xP3-ECFPafm} and pBac{3xP3-ECF*P* > >af>>}, respectively. Orientation was chosen to be opposite to that of the insulator repeats. Similar constructs containing the cytomegalovirus core promoter (nucleotides 319–438 of pUHD10-3; ref. 23) or the minimal promoter of the heat-shock gene *hsp70* (consisting of the TATA-box deleted at –43, isolated from pCaSpeRhs43AUGβgal)[36] instead of the *P* promoter did not yield functional transgenic lines, presumably because of basal promoter activity resulting in *hid*^{Ala5} expression.

***nullo-tTA* and *sryα-tTA* constructs.** *nullo*-promoters were PCR-amplified from pCNFHA38 (ref. 37) using the primers CH44R (5′-GCTCTAGA-CATTTTGAGATTTCTTGAGAATG-3′) as well as CH45F (5′-TTG-GCGCGCCTTACAGCGGGCAATTATG-3′) to yield n1 and CH46F (5′-TTGGCGCGCCTAGGGGTTAGGATTTTATGA-3′) to yield n2, respectively. *sryα* promoters were PCR-amplified from pNR*sryα* (ref. 24) using the primers CHsryaRev (5′-GCTCTAGACATGCTGTTCTATCAGATGTG-3′) as well as CHsrya2Fwd (5′-TTGGCGCGCCTTTGCTAGATTAATCTAA-GAAG-3′) to yield s1, CHsrya1Fwd (5′-TTGGCGCGCCAGTTCTTTGC-CGTTCTTC-3′) to yield s2, and CH40F (5′-TTGGCGCGCCAGTAG-GTAAAGTAGCGG-3′) to yield s3, respectively. Final *sryα* and *nullo* constructs (Fig. 1) were generated by cloning the *Asc*I- and *Xba*I-cut PCR fragments together with a 2.5 kb *Xba*I-*Fse*I fragment from pSLfa_p-*tTA*-K10_fa (ref. 34) into *Asc*I- and *Fse*I-cut pBac{3xP3-EYFPaf} (in case of *s1–s3*; *n1*, *n2*) and pBac{3xP3-EYF*P* > >af>>} (in case of >>*s1*>> and >>*s2*>>).

In addition, primers CH41F (5′-GGAATTCCGTAGCGGATTTCGC-GAATTT-3′) as well as CH42R (5′-CCGCTCGAGCTTTGCCAAGCG-CACAGGT-3′) were used to yield s4 and CH43R (5′-CCGCTCGAGGTA-GAAAGGACCTATTTTGG-3′) to yield s5, respectively. *Eco*RI- and *Xho*I-cut s4 and s5 PCR products were cloned together with a 1.7 kb *Xho*I-*Hin*dIII fragment from pSL_fa-TRE-hs43-*tTA*-SV40_fa (containing the minimal *hsp70* promoter) into *Eco*RI- and *Hin*dIII-cut pSLfa1180fa (ref. 30) to yield pSLfa_s4-*tTA*_fa and pSLfa_s5-*tTA*_fa. Final *s4* and *s5* constructs (Fig. 1) were prepared by cloning the 1.7 kb *Asc*I-fragments into *Asc*I-cut pBac{3xP3-EYFPaf}. pSL-fa-TRE-hs43-*tTA*-SV40-fa was the result of a 250 bp *Asp*718 fragment (Klenow-blunted) from pCaSpeRhs43AUGβgal cloned into *Eco*RI(Klenow-blunted), *Stu*I-cut pSLfa_TRE-*tTA*-SV40_fa. pSLfa_TRE-*tTA*-SV40_fa was constructed by cloning a 1.0 kb *Eco*RI-*Bam*HI fragment from pTet-Off (Clontech, Palo Alto, CA) into *Eco*RI-*Bam*HI-opened pSLfa_TRE-SV40_fa.

***D. melanogaster* culture.** *D. melanogaster* germline transformation using *piggyBac* vectors was done as described previously[38]. Filter sets required to identify the different fluorescence markers have been described[30]. Strains were reared under standard laboratory conditions and all tetracycline concentrations are in μg/ml of standard *D. melanogaster* medium[39], and unless otherwise stated, all incubations were at 25° C.

To analyze the efficiency of EL#42 male sterility, survivors were identified as larval progeny and the hatch rate was determined by counting the eggs in three representative vials per temperature, extrapolating the numbers. To control fertility at 29 °C, ten wild-type (Oregon R) control matings were analyzed. For competitiveness experiments, all flies were collected within 1–7 d after eclosion and pooled to average the influence of age on fecundity.

Multiplex single-embryo PCR. Embryos were dechorionated and single germband elongated embryos were homogenized in 20 μl of squishing buffer (10 mM Tris, pH 8.2; 1 mM EDTA; 25 mM NaCl; 0.2 mg/ml proteinase K). The homogenate was incubated at 50 °C for 1 h and then at 95 °C for 10 min. Five microliters of single-embryo homogenates were used for PCR reactions (3 min at 95 °C; 33 rounds of 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C; and 7 min at 72 °C). The multiplex primer mix contained (i) the primers DH2C07 (5′-AGGCCAAAGTCAGCCACCTGG-3′) and

AK/03/2C (5′-CAGGTGGAGCAGGACTGTGAT-3′) to amplify the endogenous gene *cdc2c* (ref. 40) as control for successful PCR (Fig. 5B), (ii) the primers CH_tTAm_F (5′-GGAAGATCAAGAGCATCAAGTC-3′) and CH_tTAm_R (5′-CTGTACGCGGACCCACTTTC-3′) to identify the presence of a driver transgene, and (iii) the primers CH_hidm_F (5′-CACTCGAGCAGCAGCAATAATC-3′) and CH_hidm_R (5′-TCATTCGATTACACGTCTCCTG-3′) to identify the presence of the effector transgene *TREp-hid*^{Ala5} (primers have been chosen to amplify a 253 bp fragment of the cDNA-based transgene, whereas the endogenous 2.2 kb genomic, intron-containing *hid* fragment[19] is not amplified).

Embryology. *In-situ* hybridization to whole-mount preparations of embryos was done as described[41]. RNA probes were prepared by *in vitro* transcription from pBluescriptSK-*hid*^{Ala5} and pBluescriptKS-*tTA*, which was constructed by cloning the 1.0 kb *Eco*RI-*Bam*HI fragment from pTet-Off into *Eco*RI- and *Bam*HI-cut pBluescriptKSII (Stratagene, La Jolla, CA). TUNEL labeling was done essentially as described[42], omitting immunolabeling but including a 5 min Hoechst 33258 (1 μg/ml) incorporation step after the labeling protocol.

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Competing interests statement

The authors declare that they have no competing financial interests.

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ORIGINAL PAPER

Appendix E

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Doxycycline-induced transgene expression during Drosophila development and aging

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Abstract The "reverse" tetracycline repressor (rtR) binds a specific DNA element, the tetracycline operator (tetO), only in the presence of tetracycline, or derivatives such as doxycycline (dox). Fusion of rtR to the transcriptional activation domain of herpes virus protein VP16 produces a eukaryotic transactivator protein (rtTA). rtTA has previously been shown to allow dox-dependent transcription of transgenes linked to tetO sequences in mammals. To adapt this system to *Drosophila*, the *Actin5C* promoter was used to drive constitutive expression of rtTA in transgenic flies. Three reporter constructs, each encoding *E. coli* β-galactosidase (β-gal), were also introduced into transgenic flies. In one reporter seven tetO sequences were fused to the *Adh* core promoter. The other two reporter constructs contain seven tetO sequences fused to the *hsp70* core promoter. Feeding of transgenic *Drosophila* containing the rtTA construct and any one of the three reporter constructs with dox caused up to 100-fold induction of β-gal. Dox induced β-gal expression in all tissues, in larvae and in young and senescent adults. Induction of β-gal in adults had no detectable effect on life span. These results suggest the potential usefulness of this system for testing specific genes for effects on *Drosophila* development and aging.

Key words Tetracycline repressor · Inducible promoter · Drosophila · Aging

Introduction

Inducible gene expression systems have long been an important tool in analyzing the function of specific genes in bacteria, yeasts, and *Drosophila*. In *Drosophila*, inducible transgenic systems usually rely wholly or in part on the use of a heat shock protein (hsp) gene promoter, which is transcriptionally induced in response to heat stress (Lis et al. 1983). While hsp gene promoters have been used to great advantage in many experiments, the system has several important limitations. First, the heat stress required for induction can have pleiotropic effects, including developmental abnormalities (phenocopies) (Lindquist 1986) and reduced fertility and viability. This is a problem particularly in experiments designed to study the aging process, since life span will be dramatically affected by changes in fertility and viability (Tower 1996). Another situation where the use of a heat-inducible promoter is problematic is the analysis of the heat shock proteins themselves. It is not possible to induce expression of a single hsp and study its effects, without the complication of inducing the entire endogenous repertoire of hsps. This problem has been overcome in certain experiments by using the metallothionein promoter (Petersen and Lindquist 1988; Solomon et al. 1991), which is inducible by heavy metal ions. However, the metallothionein promoter functions only in specific gut cells in transgenic *Drosophila* (Otto et al. 1987), thus limiting its potential usefulness. Therefore, there is a need for an alternative inducible gene expression system in *Drosophila*.

In the last five years, efficient inducible gene expression systems have been developed for mammalian systems based on the *E. coli* tetracycline repressor (tetR) (Gossen and Bujard 1992; Furth et al. 1994). The tetR binds to its target sequence, the tetracycline operator (tetO) only in the absence of the antibiotic tetracycline. The first system developed for mammals was the "tet-off" system (Gossen and Bujard 1992; Furth et al. 1994; Shockett et al. 1995). tetR protein was fused with the transcriptional activation domain of herpes virus transcription factor VP16. In the absence of tetracycline this protein binds to tetO sequences placed within the promoter of a gene of interest, thereby driving transcription. Addition of tetracycline then prevents binding and stops transcription ("tet-off"). A "tet-on" system was created

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by generating a mutant tetR:VP16 fusion protein, which had the reverse property of only binding to the tetO and activating transcription in the presence of tetracycline ("tet-on") (Gossen et al. 1995; Kistner et al. 1996). We report here the successful adaptation of the "tet-on" system to transgenic *Drosophila*.

Materials and methods

Plasmid constructions

Plasmid rtTA (reverse-tetracycline Trans Activator) was constructed by first inserting the 850-bp *Hin*dIII-*Xba*I fragment from pCaSpeR-AUG/β-gal (Thummel et al. 1988), containing the SV40 splice and poly(A) signals, into the *Hin*dIII (partial restriction digestion) and *Xba*I sites of the polylinker of the pCaSpeR4 transformation vector (Thummel and Pirotta 1992), to generate plasmid cSV. Plasmid pUHD172–1neo (Gossen et al. 1995) was digested with *Eco*RI, endfilled with T4 polymerase, then digested with *Bam*HI, to liberate a 1-kb fragment containing the reverse-tetracycline trans-activator coding sequence. Plasmid cSV was digested with *Spe*I, endfilled with T4 polymerase, then digested with *Bam*HI, and the 1-kb fragment from pUHD172–1neo was inserted, to generate the plasmid cTSV. DNA sequencing of cTSV revealed that it had resulted from an unexpected ligation event: the *Eco*RI site from the inserted fragment was conserved in this cloning step, and the 1-kb fragment was actually inserted into the *Bam*HI site, without any change in the *Spe*I site. The *Actin5C* promoter was inserted into plasmid cTSV in several steps. First, plasmid D237 (also called "Act5C > Draf+ > nuc-lacZ"; Struhl and Basler 1993) was digested with *Not*I, endfilled with T4 polymerase, then digested with *Kpn*I, and the resultant 4.3-kb fragment containing the *Actin5C* promoter was inserted into the *Kpn*I/*Eco*RV sites of pBlueScript II KS (Stratagene), to generate pAc. The 4.3-kb *Actin5C* promoter fragment was liberated from pAc by restriction digestion with *Kpn*I and *Eco*RI, and inserted into the *Kpn*I/*Eco*RI sites of cTSV, to generate the plasmid cATSV. DNA sequencing revealed that the *Actin5C* promoter in plasmid cATSV was in the wrong orientation relative to the reverse-tetracycline transactivator coding region. To correct this, the *Actin5C* promoter region was liberated by digestion with *Eco*RI, and then re-inserted into the same *Eco*RI site. DNA sequencing was used to identify a construct with the *Actin5C* promoter in the correct orientation, which was then named plasmid rtTA.

The seven tandem repeats of the tetO region in plasmid pUHC13–3 (Gossen et al. 1995) were amplified by PCR using the primers: 5′-TCGACTGCAGCTTTCGTCTTCAAGAATTCCTC-GAG-3′ and 5′-AGCTTCTAGATACACGCCTACTCGACCC-GGGTACCGAG-3′. The 367-bp PCR product was digested with *Pst*I and *Xba*I at the sites engineered into the primers, and then inserted into the *Pst*I/*Xba*I sites of pBlueScript II, to generate plasmid p7T.

Plasmid 7TAdh was constructed as follows. Plasmid pAdh/β-gal (Irvine et al. 1991; Koelle et al. 1991) was partially digested with *Eco*RI, and then completely digested with *Pst*I to liberate a 4.8-kb fragment containing the *Adh* basal promoter region (positions −33 to +53), the *Ubx* 5′ leader sequences fused to *lacZ*, and the SV40 splice and poly(A) signals. This fragment was cloned into the *Pst*I/*Eco*RI sites of the pCaSpeR4 polylinker, to generate plasmid pCaSpeR-Adh/β-gal. The 359-bp *Pst*I-*Xba*I fragment from plasmid p7T, containing the heptameric tetO region, was then inserted into the *Pst*I/*Xba*I sites of pCaSpeR-Adh/β-gal, to generate plasmid 7TAdh.

Plasmid 7T40 was constructed as follows. Construct c70Z (Simon and Lis 1987) was digested with *Hin*dIII and *Eco*RI to liberate a fragment containing the *hsp70* promoter fused to *E. coli lacZ*. This *Hin*dIII-*Eco*RI fragment was cloned into the *Hin*dIII/*Eco*RI sites of plasmid pBS2N to generate plasmid pBS2N′. Plasmid pBS2N is pBlueScript II KS + (Stratagene) in which the unique *Kpn*I site has been converted to a *Not*I site (a gift of L.R. Bell, University of Southern California). Construct c70Z was also digested with *Eco*RI alone to liberate an *Eco*RI fragment containing the *hsp70* poly(A) signal sequences, and this fragment was cloned into the unique *Eco*RI site of plasmid pBS2N′ to generate plasmid pBS2N″. Plasmid pBS2N″ was digested with *Hin*dIII and *Apa*I, treated with exonuclease III and with nuclease S1, and then ligated. The resultant plasmid was called c40Z, and DNA sequencing revealed a 5′ *hsp70* promoter deletion to position −40 relative to the start site of transcription. Plasmid c40Z is one of a series of *hsp70* 5′ promoter deletions which will be described in detail elsewhere (J. C. Wheeler and J. Tower, unpublished data). Plasmid c40Z was digested with *Not*I to liberate a 3.7-kb fragment containing the entire 5′Δ-40 *hsp70:lacZ* fusion gene, and this fragment was cloned into the *Not*I site of p7T, to generate plasmid p7T40-pre. A fragment containing the seven tetO repeats and the entire 5′Δ-40 *hsp70:lacZ* fusion gene was liberated from p7T40-pre by digestion with *Xho*I and *Spe*I, then inserted into the *Xho*I/*Spe*I sites in the polylinker of pCaSpeR4, to generate plasmid 7T40.

Plasmid 7TAUG was constructed as follows. A 4.6-kb SalI fragment from pCaSpeR-AUG/β-gal (Thummel et al. 1988), containing the *Adh* translation initiation sequence fused to *lacZ* and the SV40 splice and poly(A) signals, was cloned into the *Sal*I site of pBlueScript II KS, to generate plasmid pAUG. A *Pst*I fragment from plasmid 7T40, containing the seven tetO repeats and the *hsp70* promoter from −40 to +86, was inserted into the *Pst*I site of pAUG, to generate plasmid p7TAUG. A fragment containing the seven tetO repeats and the entire *hsp70:lacZ* fusion gene was liberated from p7TAUG by digestion with *Xho*I, and inserted into the *Xho*I site of pCaSpeR4, to generate plasmid 7TAUG.

Drosophila culture

Fly stocks were maintained on cornmeal/agar medium (Ashburner 1989). To obtain adult flies of defined ages, stocks were cultured at 25° C until 0–2 days post-eclosion, and then males only were transferred to 25° C or 29° C as indicated in Figure legends. These males were maintained at < 50 per vial and transferred to fresh vials every 2–4 days. Double transgenic adult males were obtained by crossing males of a transactivator stock (rtTA) to virgins of the reporter stocks (7TAdh, 7T40, and 7TAUG). Transgenic flies were generated by standard methods (Rubin and Spradling 1982), using the w^{1118} recipient strain.

Doxycycline treatments

Young flies (5–7 days post-eclosion) and old flies (28–32 days post-eclosion) were treated with the tetracycline derivative doxycycline hydrochloride (dox) (Sigma) by feeding. The indicated concentration of dox, in 20 mM Tris (pH 7.5) containing 10% sucrose, was soaked into a single Kim-Wipe (Kimberly-Clark), in an empty *Drosophila* culture vial. After feeding with dox for the specified time, the flies were returned to cornmeal/agar food vials, and allowed to recover as indicated. For treatment of larvae, the cornmeal/agar medium was supplemented with dox to a final concentration of 0.25 mg/ml, prior to seeding of the culture.

Spectrophotometric assay of β-galactosidase activity

β-Galactosidase (β-gal) activity was quantitated in whole fly extracts using published procedures (Simon and Lis 1987). Assays were performed under conditions in which the reaction was linear with regard to the amount of extract. Data are presented as the average +/− the standard deviation for triplicate assays. Protein concentration of extracts was determined using the Bradford reagent (BioRad). The w^{1118} strain was used to generate all transgenic lines, and no β-gal activity was detectable in extracts of the w^{1118} strain using the spectrophotometric assay.

In situ staining for β-galactosidase activity

β-galactosidase expression was visualized in dissected flies, larvae, and cryostat sections using published procedures (Simon et al. 1985).

Results

Basic components of the system

To achieve tetracycline-inducible induction of transgenes in all tissues it is necessary that the reverse tetracycline transactivator (rtTA) be expressed in all tissues. The rtTA is a fusion of the reverse tetracycline receptor (rtR), which binds to DNA only in the presence of tetracycline, with the transcriptional activation domain of herpes virus transcription factor VP16. In construct rtTA, the constitutive *Drosophila Actin5C* promoter was used to drive expression of the rtTA coding region. This construct also contains the SV40 poly(A) signal sequence (Fig. 1A). To test the system, three reporter constructs were generated, each encoding *E. coli* β-gal. The constructs differed in the source of the core promoter, 5′ UTR, and polyadenylation signal sequences in order to maximize the chances of generating a construct which could yield high-level transgenic protein expression in *Drosophila*. In the first reporter construct (7TAdh) seven tetO sequences are fused to the *Adh* core promoter, followed by the *Ubx* 5′ untranslated region, the *E. coli lacZ* coding region, and the SV40 poly(A) signal (Fig. 1B). A regulatory element composed of seven tetO sequences was chosen because this element was previously shown to function in transgenic mice (Kistner et al. 1996). In the second reporter (7T40), the seven tetO sequences are fused to the *hsp70* core promoter, and *hsp70* 5′ untranslated region, followed by the *E. coli lacZ* coding sequences and the *hsp70* poly(A) signal (Fig. 1C). In the third construct (7TAUG), the seven tetO sequences are fused to the *hsp70* core promoter, followed by the *Adh* 5′ untranslated region, the *lacZ* coding region, and the SV40 poly(A) signal (Fig. 1D). Multiple independent transgenic lines were generated for each construct. Each line is homozygous for the transgenic construct, and is designated by the name of the construct followed by the chromosome in which the construct is inserted (in parenthesis), followed by a letter/number combination for each independent transgenic line. For example, line 7TAdh(2)A2 is transgenic line number A2 and has the 7TAdh construct inserted on the second chromosome.

Flies were then generated which contained both the rtTA construct and the 7TAdh construct ("double-transgenic" flies). This was done by crossing flies of stock rtTA(2)C1 to flies of stock 7TAdh(2)A2, which yields progeny containing one copy of each construct. A sample of these double transgenic flies were fed sucrose solution containing 1.0 mg/ml dox for 48 h, while the controls were fed sucrose solution alone. The flies were allowed to recover for 3 days, then sectioned using a cryostat, and the sections were stained for β-gal activity (Fig. 2A). In the treated flies robust β-gal activity (blue stain) was detected in all tissues. In the control flies, low-level β-gal activity was detected primarily in the gut, and thus the system allows dox-induced transgene expression in all tissues of the adult. The same results were obtained with transgenic flies containing the other two reporter constructs, 7T40 and 7TAUG (data not shown).

To determine if the system also works during development, line rtTA(2)C1 was crossed again to reporter line 7TAdh(2)A2, and also to reporter line 7T40(3)B1, and the larvae from each cross were cultured on food containing 0.25 mg/ml dox, and on

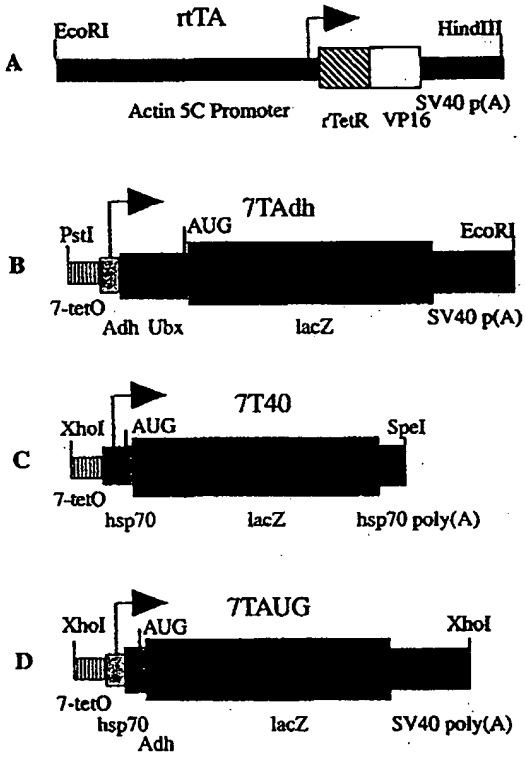

Fig. 1A–D Transgenic constructs. Each construct fragment shown is cloned into the indicated restriction sites of the polylinker of the pCaSpeR-4 transformation vector. The assembly of each construct is described in detail in Materials and methods. Diagrams are not to scale. A rtTA. The constitutive *Actin5C* promoter and 5′ untranslated region are fused to the coding sequences for the rtTA (reverse tetracycline transactivator), which is a fusion of the rtR (reverse tetracycline repressor) and the transcriptional activation domain of herpes virus protein VP16. The poly(A) signal sequences are from SV40. B 7TAdh. Reporter construct consisting of seven tetO sequences, the *Adh* core promoter, the *Ubx* 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region and the SV40 poly(A) signal sequences. C 7T40. Reporter construct consisting of seven tetO sequences, the *hsp70* core promoter, 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region and the *hsp70* poly(A) signal sequence. D 7TAUG. Reporter construct consisting of seven tetO sequences, the *hsp70* core promoter, the *Adh* 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region, and the SV40 poly(A) signal sequence

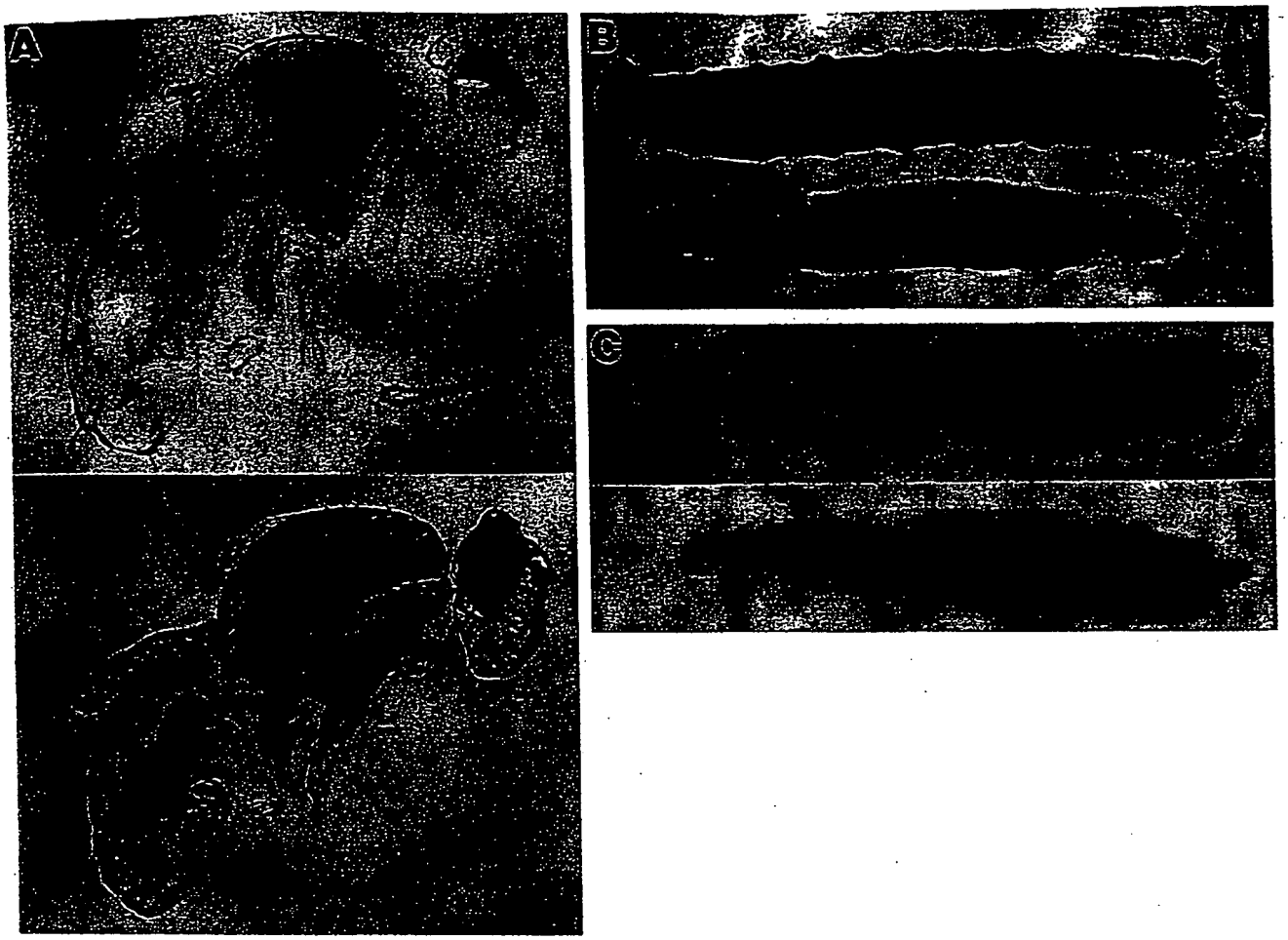

Fig. 2A–C Dox-induced transgene expression detected by an in situ β-gal activity assay. A rtTA transgenic line rtTA(2)C1 was crossed to reporter line 7TAdh(2)A2. Young adult progeny were fed with either control sucrose solution (*upper panel*) or sucrose solution containing 1.0 mg/ml dox (*lower panel*) for 48 h, and then allowed to recover for 3 days. Flies were sectioned on a cryostat, and stained for β-gal activity using the chromogenic substrate X-gal. In the control (*upper panel*), low-level β-gal activity is detected primarily in gut tissues. The gut staining indicates some leakiness of expression in the absence of dox, as in non-transgenic *Drosophila* only very faint gut staining is detectable, and only in the abdomen (data not shown, see also Wheeler et al. 1995). In dox-treated (*lower panel*), β-gal activity is detected in all tissues, with the exception of the central region of the indirect flight muscles. All of the indirect flight muscle tissue stains intensely if the staining reaction is allowed to continue for a longer period (data not shown). However with longer staining times the increased intensity of stain in the other body segments obscures the detail of specific tissues, and therefore the results for the shorter staining time are presented. B Progeny from the cross rtTA(2)C1 × 7T40(3)B1 were cultured on standard *Drosophila* culture media (upper larva) or *Drosophila* media containing 0.25 mg/ml dox (lower larva). Whole third-instar larvae were stained in situ for β-gal activity. No β-gal activity was detected in the control tissue larvae (*upper larvae*), or in non-transgenic larvae (data not shown). General β-gal activity was detected in the dox treated larvae (*lower larvae*). C Repeat of the experiment in B, using progeny of the cross rtTA(2)C1 × 7TAdh(2)A2. β-Gal expression in larvae with this reporter was reproducibly less efficient than in the experiment shown in B

control food. As seen in Fig. 2B, C, staining of whole third-instar larvae revealed high-level, tissue general induction of β-gal activity with reporter 7T40(3)B1, and somewhat lower level, tissue general induction with reporter 7TAdh(2)A2. The dox-fed larvae were also observed to be slightly smaller than the controls, which may be due to a toxic effect of the dox and/or β-gal expression during development.

Characterization of the response

The induction of β-gal expression can be quantitated by spectrophotometric assay of β-gal activity in fly extracts. This assay was used to optimize the time course of dox treatment. Transactivator line rtTA(2)C1 was crossed to reporter line 7TAdh(3)D1, and the double transgenic progeny were treated with 1.0 mg/ml of dox for 24 h, 48 h, and 48 h plus varying times of recovery without dox. As seen in Fig. 3A, 48 h of treatment plus 3 days of recovery gave the optimal degree of induction (~10-fold). With greater times of recovery, β-gal activity decreased, indicating that the induction is reversible upon withdrawal of dox. The same result was obtained using a different reporter stock, containing the 7T40

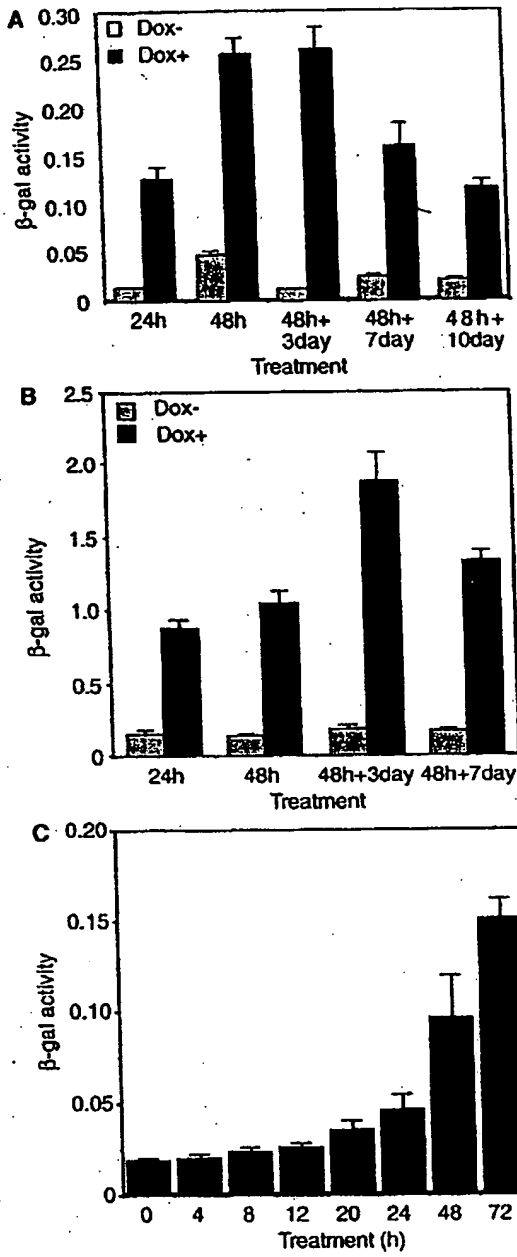

Fig. 3A–C Time course of transgene induction by dox. A Young adult progeny of the cross rtTA(2)C1 × 7TAdh(3)D1 were mock treated (Dox-; *stippled bars*), or treated with 1.0 mg/ml dox (Dox+; *black bars*) for the indicated time periods, and allowed to recover as indicated. Triplicate samples containing three flies each were homogenized, and β-gal activity was quantitated using the spectrophotometric assay. β-Gal activity is expressed in relative units, and the averages +/- SD are presented. B The experiment in A was repeated with a different reporter stock, using young adult progeny from cross rtTA(2)C1 × 7T40(2)E1. C The experiment in A was repeated using progeny from cross rtTA(2)C1 × 7TAdh(2)A2, and the timecourse for induction was analyzed in greater detail

construct, 7T40(2)E1 (Fig. 3B). Thus, both the *hsp70* core promoter and the *Adh* core promoter can respond to activation by the tetO sequences and the rtTA transactivator.

In transgenic mice the activation by the rtTA transactivator can be quite rapid, with activation by several orders of magnitude occurring in the first 4 h, and maximum levels of activation being achieved by 24 h (Kistner et al. 1996). The timecourse of activation in *Drosophila* was analyzed in greater detail (Fig. 3 C), and found to be significantly slower. In the progeny of the cross rtTA(2)C1 × 7TAdh(2)A2, induction of β-gal by dox feeding was quantitated at intervals between 4 and 72 h. Significant activation was not detected until 8–20 h, and maximal induction required ≥72 h. Similar results were obtained with construct 7TAUG (data not shown). Note that while the level of induction and timecourse was similar for the different reporters in Fig. 3, they are not identical. This probably reflects small differences in the activities of the different reporter insertions, as well as the variability inherent in working with live adult *Drosophila* and administration of dox by feeding.

The *Drosophila* tet-on system was next characterized for the dose response to dox (Fig. 4). Double transgenic adults (rtTA(2)C1 × 7TAdh(2)A2) were fed dox for 48 h and allowed to recover for 3 days (Fig. 4, open circles), or for 96 h plus a 3-day recovery period (closed circles). For 48-h treatment times, β-gal activity was found to increase in response to dox concentrations from 0.01 to 2 mg/ml. Use of the longer 96-h treatment time allowed equivalent levels of β-gal expression with one-tenth as much dox. Thus, longer treatment times reduce the amount of dox required for efficient induction.

To compare the relative activities of the three different reporter constructs, two independent transgenic lines for each reporter were crossed to the rtTA(2)C1 transactivator line (Fig. 5A). Dox-induced β-gal expression

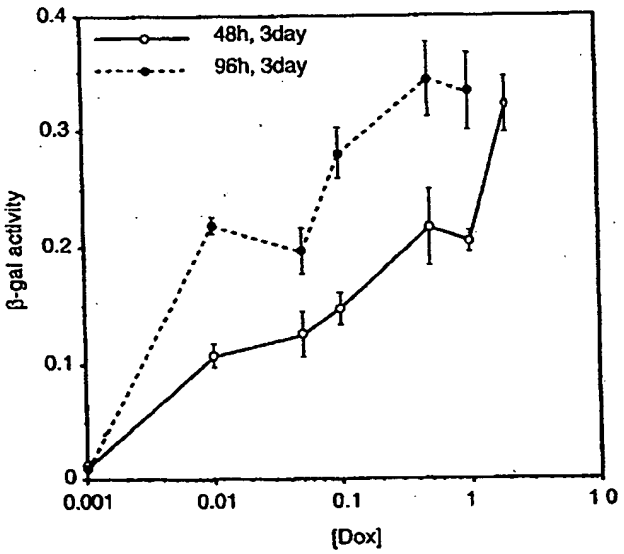

Fig. 4 Dose response of transgene induction by dox. Young adult progeny of cross rtTA(2)C1 × 7TAdh(2)A2 were fed the indicated concentrations of dox for 48 h and allowed to recover for 3 days (*open circles*), or for 96 h plus a 3-day recovery period (*closed circles*). β-Gal expression was quantitated as in Fig. 3

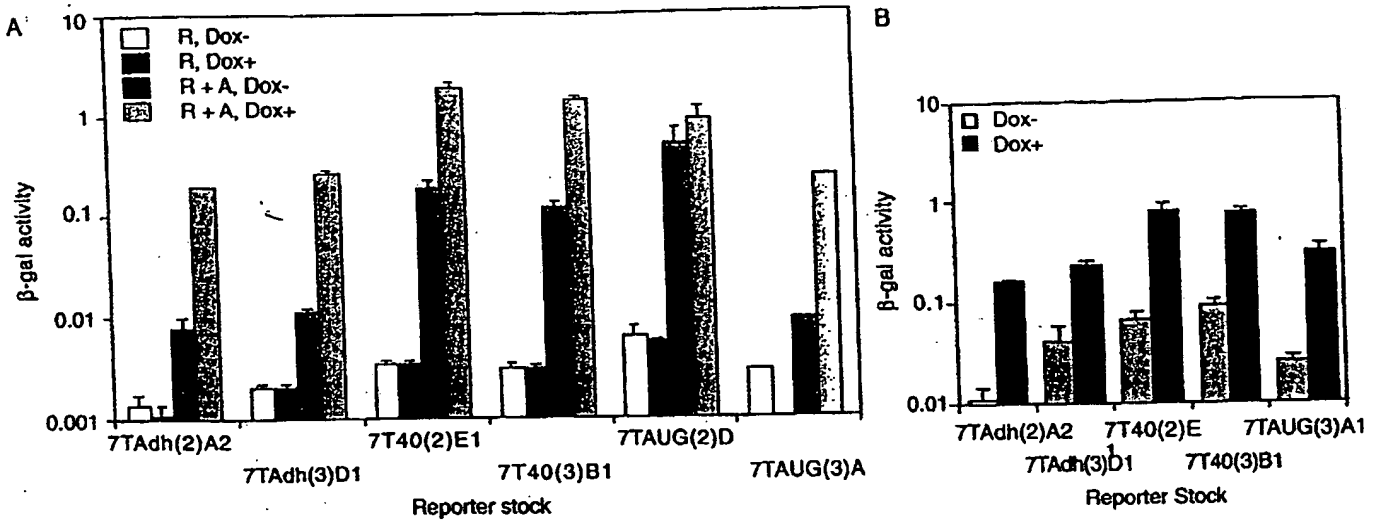

Fig. 5A, B Comparison of transgenic reporter constructs and lines. All dox treatments were for 48 h plus 3 days recovery. A Assay in young adults. The indicated reporter lines (R, reporter alone) were assayed with and without dox treatment, as indicated. Each indicated reporter line was also crossed to the rtTA line rtTA(2)C1, and the progeny (R + A, reporter plus activator) were assayed with and without dox treatment, as indicated. B Assay in old adults. rtTA line rtTA(2)C1 was crossed to each indicated reporter line, and old adult progeny were assayed with and without dox treatment, as indicated. β-Gal expression was quantitated as in Fig. 3

was observed with all three constructs, with induction factors ranging from 12- to 25- fold. In general, the 7T40 reporter construct gave higher levels of β-gal expression than the other two reporter constructs; however, the background expression in the absence of dox was also higher. Thus, the induction factor achieved was similar for each of the three reporter constructs.

To determine if the system functions during aging of *Drosophila*, the activity of each reporter construct was also assayed in senescent (30-day-old) flies (Fig 5B). Each reporter was found to support dox-induced β-gal expression in senescent flies, with induction factors ranging from 8- to 15-fold.

The dox-inducible system is dependent upon efficient, general expression of the transactivator construct, rtTA. Because the chromosomal site of insertion of the rtTA transgene can affect the level of expression, different independent rtTA transgenic lines may vary in their activity. To compare their activities, each of 13 independent rtTA transgenic lines was crossed to the 7TAdh(3)D1 reporter, and the efficiency of induction of β-gal by dox was quantitated (Fig. 6A). The different independent rtTA lines were found to vary in activity, both with regard to the amount of background β-gal activity in the absence of dox, and with regard to the maximum level of induction in the presence of dox. Transgenic transactivator line rtTA(3)E2 appeared to be the best: in the absence of dox, background β-gal levels were as low as in flies carrying the reporter construct in the absence of any transactivator, and dox treatment yielded a 40-fold induction. To confirm this result, each

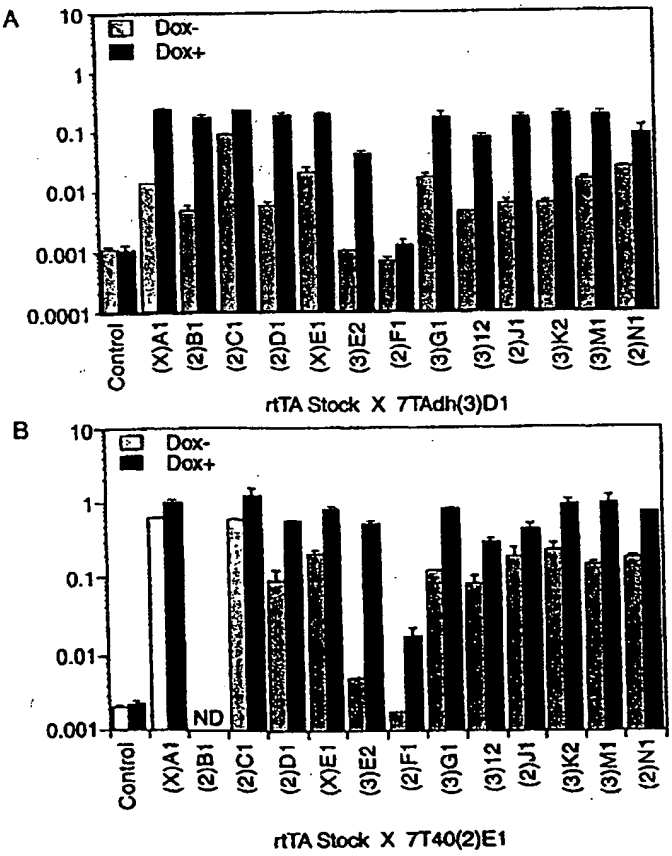

Fig. 6A, B Comparison of different transgenic transactivator (rtTA) lines. All dox treatments were for 48 h plus 3 days recovery. A The indicated rtTA lines were each crossed to reporter line 7TAdh(3)D1. The young adult progeny from each cross were assayed without dox treatment (Dox-; *stippled bars*), and with 1.0 mg/ml dox treatment (Dox+; *black bars*), as indicated. Control was the reporter line 7TAdh(3)D1 alone. B The experiment in A was repeated using the reporter line 7T40(2)E1. ND, not done

transactivator stock was also tested in combination with reporter stock 7T40(2)E1 (Fig. 6B). Again the various transactivator lines varied with regard to background and maximal level of induction, and their activity relative to each other was similar to that observed using the 7TAdh reporter. Line rtTA(3)E2 was again found to have the lowest background, and to be the most active, yielding 100-fold induction of β-gal in response to dox.

One potential use of the dox-inducible system in *Drosophila* is in the analysis of the effects of specific genes on the aging process. Ideally, for such experiments, the system itself should not have any effect on life span. To characterize the system for effects on life span, several genetic backgrounds were tested for longevity, with and without dox feeding. Wild-type flies exhibited no negative effects on life span when fed with 0.1 mg/ml dox (Fig. 7A). Double transgenic flies (rtTA(2)C1 × 7TAdh(2)A2) expressed high levels of β-gal in response to 0.1 mg/ml dox (Fig. 4), and this expression also had no detectable negative effects on lifespan (Fig. 7A). Finally, a different combination of transactivator and reporter were tested. Transactivator rtTA(X)A1 was crossed to reporter 7TAdh(3)D1 and age-synchronized cohorts of adult flies were treated throughout their adult lifespan with no dox, 0.01 mg/ml dox, or 1.0 mg/ml dox (Fig. 7B). Treatment with 1.0 mg/ml dox was found to have a negative affect on life span. However, treatment with 0.01 mg/ml dox had no detectable affect on life span. The same results were obtained with several other combinations of rtTA and reporter lines (data not shown). Since 0.01 mg/ml dox and 0.1 mg/ml dox allow high-level induction of β-gal (Fig. 4), and have no detectable effect on lifespan (Fig. 7), these results suggest that the system should be useful for assaying the effects on life span of overexpression of specific genes.

Discussion

The hybrid transcriptional activator (rtTA) consisting of the rtR fused to the transcriptional activation domain of the herpes virus protein VP16 has previously been shown to be capable of supporting dox-induced transcription in transgenic mice. The experiments presented

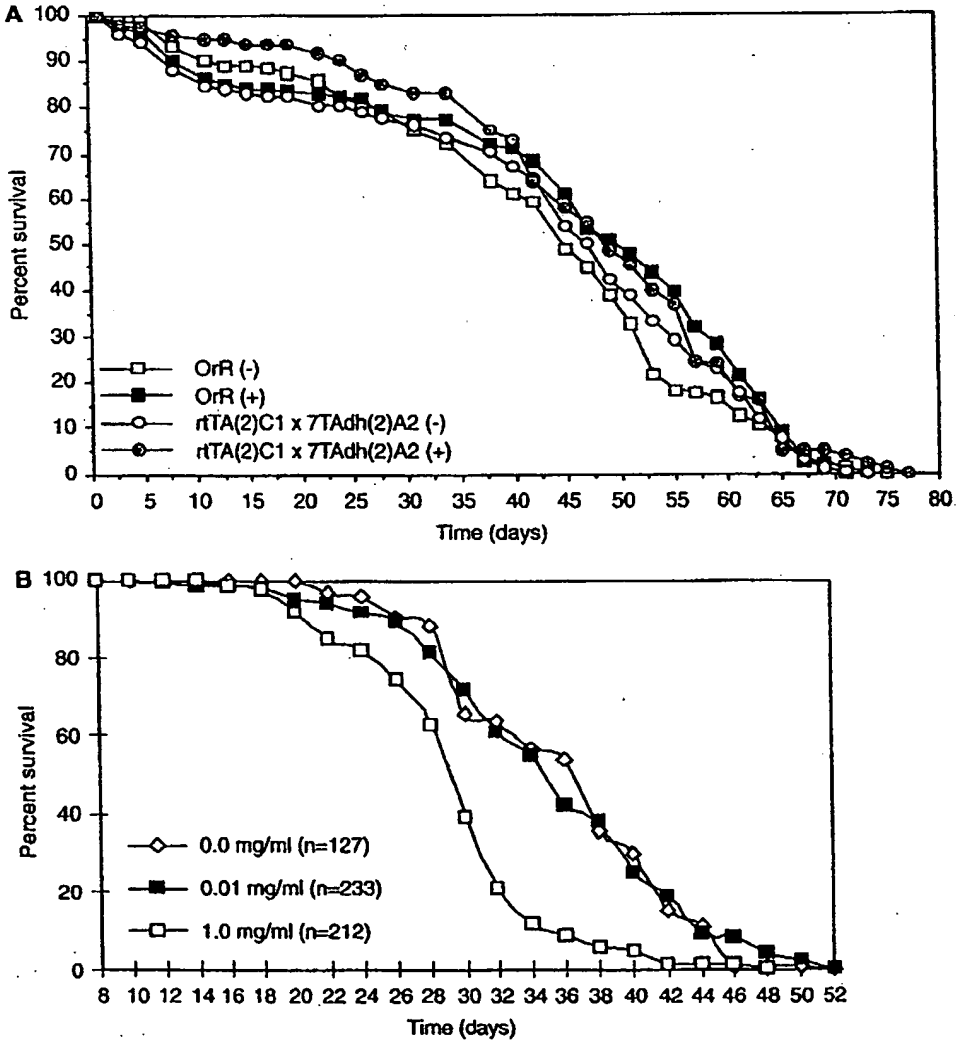

Fig. 7A, B Affect of dox-induced β-gal expression on *Drosophila* adult life span. **A** Wild-type Oregon R strain flies were treated throughout their adult lifespan either without dox (*open squares*) or with 0.1 mg/ml dox (*filled squares*), at 25° C. Flies were fed or mock-fed dox for 2 days, and then allowed to recover on standard media for 2 days, and this regimen was repeated until all the flies had died. The percentage of flies surviving is plotted as a function of time in days. The same experiment was performed using progeny from cross rtTA(2)C1 × 7TAdh(2)A2 grown in the absence of dox (*open circles*) or in the presence of 0.1 mg/ml dox (*closed circles*). At least 200 flies were used for each of the four survival curves. **B** Progeny from the cross rtTA(X)A1 × 7 TAdh(3)D1 were grown throughout their adult life span in the absence of dox (*open diamonds*), or in the presence of 0.01 mg/ml dox (*filled squares*), or 1.0 mg/ml dox (*open squares*), at 25° C. The number of flies used (n) for each survival curve is indicated. The different genotypes assayed in A and B vary in life span relative to each other, which is not unexpected due to the large effects of genetic background on life span (Curtsinger et al. 1995; Tower 1996)

here demonstrate that this rtTA functions in transgenic *Drosophila*, and can activate transcription at both the *hsp70* and *Adh* core promoters when they are linked to tetO sequences. Since all three reporter constructs performed similarly, the results suggest that in this system there is no significant difference in the effectiveness of the SV40 poly(A) signal relative to the *hsp70* poly(A) signal, and no significant difference between the effectivness of the *Adh*, *hsp70* and *Ubx* 5′ UTR regions. Dox-induced transgene expression was detected in all tissues, and induction ranged from 10- to 100-fold. Different transgenic lines containing the rtTA construct varied considerably in activity. Variation was observed in the maximal level of induction achieved, and in the amount of background activity observed in the absence of dox. This variation is likely to be due to chromosomal position effects on the expression of the rtTA transposon, and perhaps to other differences in the genetic background of the lines. The different transgenic lines of the reporter constructs also varied in activity, most probably for the same reasons. The maximum degree of induction that was achieved was 100-fold. This is dramatically less than the five orders of magnitude induction obtained with the tet-on system in transgenic mice (Kistner et al. 1996). The maximal induction achieved in *Drosophila* is limited by at least two factors: First, the reporter constructs are slightly leaky, in that variable, low-level β-gal activity is detected even in the absence of the rtTA transactivator. Second, the rtTA transactivator appears to be partially active even the absence of dox treatment, in that reporter plus transactivator was often more active than reporter alone. The first problem might be addressed by protecting the reporter constructs from position effects with insulator elements (Roseman et al. 1993), and/or by identifying a less leaky core promoter. The second problem can be mitigated by identifying particular rtTA lines, such as rtTA(3)E2, which exhibit less background activation. Finally, we hypothesize that the herpes virus VP16 transcriptional activation domain used to create the rtTA transactivator may be better suited to interaction with the mammalian transcriptional machinery than with the *Drosophila* transcriptional machinery. This possibility may also be relevant to the slower time course of induction observed in *Drosophila*.

Despite its limitations, this inducible system has several potential advantages relative to the use of heat shock gene promoters. The dox-inducible system should be useful for studying hsps, as it will allow the investigator to induce the expression of a single hsp, and potentially inhibit its expression with antisense RNA, without inducing the endogenous heat shock response. The dox- inducible system also allows the investigator to induce a gene of interest at any time during the life cycle. This is particularly relevant to study of the aging process (Curtsinger et al. 1995; Tower 1996), where it is often desirable to alter gene expression specifically in the adult. For example, constitutive over-expression of Cu/Zn SOD may have beneficial effects on *Drosophila* life span but it also appears to have toxic affects during pupal development (Reveillaud et al. 1991). Using the dox-inducible system it should be possible to avoid toxic effects during development and cause over-expression of transgenes specifically in the adult where beneficial effects on life span may be more apparent.

The dox-inducible system should be readily adaptable to tissue-specific induction. Replacement of the constitutive *Actin5C* promoter in the transactivator construct rtTA with a tissue-specific promoter should provide tissue-specific expression of the rtTA transactivator and thus tissue-specific induction of the reporter. An elegant system for tissue-specific expression of the yeast GAL4 transactivator has been developed for *Drosophila* (Brand and Perrimon 1993; Brand and Dormand 1995). In this case tissue-specific expression of the GAL4 transactivator is driven by an "enhancer-trap" system: the transactivator is under the control of a weak transcriptional promoter which can become activated in a tissue- and temporal-specific manner when the P element inserts near transcriptional enhancer sequences in the chromosome. The large variety of tissue- and temporal-specific GAL4 transactivator expression patterns generated thus allows tissue- and temporal-specific expression of "reporter" type constructs containing GAL4 binding sites in their promoters. This system could be adapted to drive expression of the rtTA transactivator, thus creating a large variety of tissue-specific expression patterns inducible by dox.

Finally, it may be possible to create dox-dependent mutations in *Drosophila*. P element constructs with transcriptional promoters directed out of the end of the P element can cause over-expression and/or mis-expression of genes near the site of insertion, sometimes causing dominant mutations (Rorth 1996; Hay et al. 1997). Creation of a P element with a dox-inducible promoter directed out of the P element into flanking DNA sequences should sometimes cause dox-induced over-expression of a gene near the insertion site. This method should thus yield conditional (dox-dependent), dominant, gain-of-function mutations which would be useful for many types of genetic analyses; such experiments are now underway.

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High-Frequency Generation of Conditional Mutations Affecting *Drosophila melanogaster* Development and Life Span

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ABSTRACT

Genome sequencing reveals that a large percentage of Drosophila genes have homologs in humans, including many human disease genes. The goal of this research was to develop methods to efficiently test Drosophila genes for functions *in vivo*. An important challenge is the fact that many genes function at more than one point during development and during the life cycle. Conditional expression systems such as promoters regulated by tetracycline (or its derivative doxycycline) are often ideal for testing gene functions. However, generation of transgenic animals for each gene of interest is impractical. Placing the doxycycline-inducible ("tet-on") promoter directed out of the end of the *P* transposable element produced a mobile, doxycycline-inducible promoter element, named *PdL*. *PdL* was mobilized to 228 locations in the genome and was found to generate conditional (doxycycline-dependent), dominant mutations at high frequency. The temporal control of gene overexpression allowed generation of mutant phenotypes specific to different stages of the life cycle, including metamorphosis and aging. Mutations characterized included inserts in the *α-mannosidase II* (*dGMII*), *ash1*, and *pumilio* genes. Novel phenotypes were identified for each gene, including specific developmental defects and increased or decreased life span. The *PdL* system should facilitate testing of a large fraction of Drosophila genes for overexpression and misexpression phenotypes at specific developmental and life cycle stages.

SEQUENCING of the Drosophila genome reveals ~13,600 genes (Adams *et al.* 2000). At least half of these are homologous to human genes, including more than half of known human disease genes (Rubin *et al.* 2000). The powerful molecular and genetic tools available for Drosophila have made this organism a highly successful model for determining the functions of conserved genes. However, such research is often labor intensive and new functional genomic research methods are required to study the large number of interesting genes identified by the genome sequence. A critical obstacle to this research is the fact that many genes function in multiple processes, at multiple stages of the life cycle, and may be required for viability. Simple loss-of-function mutations or gene "knockouts" often do not allow the organism or cells to proceed to the stage of interest. Conditional mutations, such as ones expressed only at permissive temperatures, overcome this obstacle by providing temporal control of gene function. Temperature-sensitive mutations have been essential for the analysis of diverse biological processes including yeast and mammalian cell cycle and Drosophila and *Caenorhabditis elegans* development. However, temperature-sensitive mutations are not ideal because they are rare and the temperature shift often has confounding effects on other genes and processes. For example, temperature-sensitive mutations are particularly problematic for study of Drosophila aging, as life span is profoundly affected by temperature (Baker *et al.* 1989).

Conditional gene expression systems provide a type of conditional, dominant misexpression "mutation." Gene expression can be activated with temporal control in transgenic animals using systems based on recombination and systems triggered by hormones or other chemicals. For example, tetracycline [or doxycycline (DOX)]-regulated promoters have been used to facilitate numerous studies of gene function in mammals and, more recently, in Drosophila (Gossen and Bujard 1992; Gossen *et al.* 1995; Bello *et al.* 1998; Bieschke *et al.* 1998). Conditional systems are particularly well suited to the study of aging (Bieschke *et al.* 1998; Sun and Tower 1999; Tower 2000; J. Sun, D. Folk, T. J. Bradley and J. Tower, unpublished data). They allow the investigator to avoid toxic or other effects of gene expression during development and determine gene effects in the aging adult, such as increased or decreased life span. In addition, like other quantitative traits, life span is sensitive to genetic background. Conditional systems provide powerful controls for genetic background, as control and overexpressing animals are genetically identical. A drawback of this approach is that generation of transgenic animals for each gene of interest is labor intensive and is not practical for large scale functional genomics.

The Drosophila *P* transposable element can be readily

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mobilized to generate hundreds or thousands of Drosophila lines with unique insertions (Cooley *et al.* 1988). The *P* element has been engineered to facilitate many types of genetic manipulation, including insertional mutagenesis, enhancer-trapping, and generation of gene misexpression mutations (O'Kane and Gehring 1987; Bellen 1989; Spradling *et al.* 1995; Rorth 1996; Hay *et al.* 1997; Rorth *et al.* 1998; Toba *et al.* 1999; Lucasovich *et al.* 2001). In the experiments presented here the usefulness of *P*-element mutagenesis has been extended by engineering a *P* element (*PdL*) that creates conditional (DOX-dependent) mutations at high frequency. The system combines the benefits of the tet-on conditional gene expression system with the utility of *P*-element mutagenesis.

MATERIALS AND METHODS

Drosophila strains: All *D. melanogaster* strains are as described (Lindsley and Zimm 1992; http://flybase.bio.indiana.edu/).

Drosophila culture and life-span assays: Drosophila were cultured on standard agar/molasses/cornmeal/yeast media (Ashburner 1989). To obtain adult flies of defined age, the indicated *PdL* lines and Oregon-R control strain were crossed to *rtTA* stock and cultured at 25° in urine specimen bottles. Prior to eclosion of the majority of pupae, bottles were cleared of adults and newly eclosed flies were allowed to emerge over the next 48 hr. The majority of the males will have mated during this time. The males only were then removed and were designated 1 day old and were maintained at 25° at 40 per vial in culture vials with food. At 4 days of age the males were split into control and experimental groups of 200 males each, with experimentals (+DOX) placed on culture media supplemented with 250 μg/ml DOX. Dead flies were counted at each passage, and the number of vials was progressively reduced to maintain ~ 40 flies per vial. To calculate mean life spans for the experimental (+DOX) and control (−DOX) cohorts, each fly's life span was tabulated, their life spans were averaged, and the SEM was calculated. Statistical significance of differences in mean life span was calculated for each experiment using unpaired two-sided *t*-tests.

Construction and transformation of *PdL*: A 560-bp *Eco*RI to *Pst*I fragment, containing seven *tetO* repeats and the *hsp70* core promoter from −40 to +86, was excised from plasmid p7T40 (Bieschke *et al.* 1998) and cloned into the polylinker of pCaSpeR-4 transformation vector (Thummel and Pirotta 1992). Multiple independent germ-line transformants of the *PdL* construct were generated using standard methods (Rubin and Spradling 1982), using the *y-ac-w*^{1118} recipient strain (Patton *et al.* 1992).

Southern analysis of *PdL* copy number: DNA was isolated from *PdL* lines and restriction digested with *Xba*I, *Hin*dIII, and *Pst*I. DNA was transferred to Southern blot and hybridized with a radiolabeled 172-bp fragment from the 3′ P end of *PdL*. This probe fragment was generated by PCR amplification with primers located within the 3′ P end, IRREV (atgatgaaataaca taaggtggtcccg) and P3MCSREV (atgagttaattcaaaccccacggacat).

Inverse PCR amplification of *PdL* flanking sequences: Protocols were as previously described (Tower *et al.* 1993; Tower and Kurapati 1994). Briefly, DNA equivalent to one fly was restriction digested with either *Xba*I, *Hin*dIII, or *Pst*I. The DNA was extracted with phenol/chloroform, precipitated with ethanol, resuspended, and treated with T4 ligase overnight at 16°. PCR amplification was performed using primers Pry1 (ccttagcatgtccgtgggggtttgaat) and IR (cgggaccaccttatgttatttcat catg). PCR protocol was as follows: step 1, 95° for 5 min; step 2, 95° for 30 sec; step 3, 51° for 1 min; step 4, 72° for 1 min; step 5, repeat steps 2–4, 40 times; step 6, 72° for 10 min. The PCR product was subcloned into the pCR2.1-TOPO cloning vector (Invitrogen, San Diego). Dideoxy sequencing was carried out using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical, Cleveland) and the T7 and M13 reverse sequencing primers.

DNA sequence analyses: *PdL* flanking DNA sequences were used to query GenBank databases using BLASTN program with default settings as provided at the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/).

Northern analyses: Messenger RNA was isolated from adult Drosophila using the RNAqueous kit (Ambion, Austin, TX), fractionated on 1.0% agarose gels and transferred to Gene Screen membranes (DuPont/NEN, Boston, MA). The DNA probe for exon 4 of the *dGMII* gene was generated by PCR amplification from Drosophila genomic DNA using primers GMIIFWD (ctcgtatgcatcgaatctcttgc) and GMIIREV (ttggccga gctcttggttaagc). The dGMII intergenic region probe was generated using primers GL178478-5 (ggataaagcagaaactgaagccaag) and GL178890-3 (gcagttgcgtcattatcactaagcc). The CG16765 open reading frame (ORF) probe was generated using primers GL179311-5 (cagaatgcaatcttatcagctccag) and GL179719-3 (tggc gaccacgatgatctgaatct). The probe for exon 1 of the *ash-1* gene was generated using primers ASH1FWD (gcgcaagaagctgg caag) and ASH1REV (tgtatcgactcttgcagctggcat). The probe for *pum* exon 9 + 10 was a 700-bp *Eco*RI to *Sac*I fragment of Drosophila cDNA clone SD03602 (Genome Systems). The probe for *pum* intron 8 was generated using line 3B2 genomic DNA template and primers IR (cgggaccaccttatgttatttcatcatg) and pum120121 (gtgaaacatttagcttcgacggatgt). The loading control was ribosomal protein gene *Rp49* (O'Connell and Rosbash 1984). DNA probes were ^{32}P-labeled using the Prime-It II DNA labeling kit (Stratagene, La Jolla, CA). Hybridization signals were visualized by autoradiography. Transcript size was determined by comparison with 1-kb RNA ladder (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's instructions.

Electron microscopy: Scanning electron microscopy was carried out at the University of Southern California Center for Electron Microscopy and Microanalysis, using a Cambridge 360 SEM. Samples were prepared using standard methods, except that critical point drying was replaced by a 15-min treatment with hexamethyldisilazane (Adams *et al.* 1987).

RESULTS

Construction, transformation, and transposition of the novel *P*-element mutagen *PdL*: The tet-on tetracycline inducible system (Gossen *et al.* 1995) was recently adapted to transgenic Drosophila (Bieschke *et al.* 1998). The cytoplasmic actin (*actin5c*) promoter was used to drive constitutive, tissue-general expression of the *r*everse *t*etracycline *t*rans *a*ctivator (rtTA) in a transgenic construct called *rtTA*. A tetracycline(DOX)-inducible promoter was constructed by placing seven repeats of the rtTA binding site ("tetO") upstream of a core promoter. The core promoter consisted of *hsp70* gene sequences from −40 to +86, including the TATAA box, the transcriptional initiation site, and 86 bp of 5′-untranslated region (UTR) sequences. The *hsp70* core

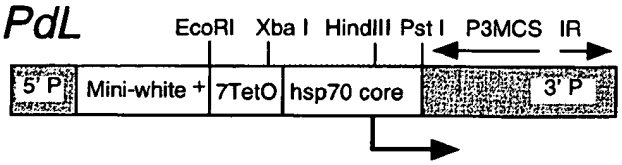

FIGURE 1.—P-element construct *Ponce de Leon* (*PdL*). The 5′ and 3′ P end sequences required for transposition are indicated by stippled boxes. Restriction sites and primers used in inverse PCR cloning of flanking sequences are indicated. The orientation of the DOX-inducible synthetic promoter is indicated by a bent arrow. The figure is not to scale.

promoter lacks all heat-shock response elements and is therefore no longer responsive to stress or age (SIMON *et al.* 1985; WHEELER *et al.* 1999). The synthetic promoter was cloned into the pCaSpeR-4 transformation vector (THUMMEL and PIROTTA 1992), with the promoter directed out through the 3′P end sequences to generate *PdL* (Figure 1). *PdL* was transformed into Drosophila using standard methods to create 13 independent transgenic lines.

Three of the *PdL* transformant lines were tested for frequency of transposition by appropriate crosses to Δ2-3 transposase source (ROBERTSON *et al.* 1988). Line *PdL*(X)A yielded the highest frequency (27%) and was used to generate 215 new insertions on the second and third chromosomes, using standard methods (COOLEY *et al.* 1988; TOWER and KURAPATI 1994).

Identification of conditional, dominant mutations affecting metamorphosis: The 215 new insertions of *PdL* were analyzed along with the 13 original transformant lines. Each of the total 228 *PdL* insert lines were crossed to flies containing the *rtTA* transactivator construct, using culture media ±DOX. DOX was not present inside the eggshell because the mothers were not prefed DOX. In this way gene misexpression will be specific to the larval and pupal stages. Progeny containing both *PdL* and *rtTA* were scored for viability and visible phenotypes. Out of 228 *PdL* lines, nine lethal and five visible mutations were identified (Table 1). As expected, the mutations were both conditional (DOX dependent) and dominant. Visible phenotypes included curled wings, blistered wings, and rough eyes (Figure 2). For each of the five visible mutations, zero flies exhibited the mutant phenotype in the absence of DOX. Therefore there is no detectable leakiness of this system with regard to mutant phenotypes. For both the visible and lethal mutations in the presence of DOX, penetrance of the phenotype was high, and varied from 67 to 100%, with most at 100% (Table 1).

Each of the *PdL* insertions was made homozygous in the absence of *rtTA* to assay for recessive phenotypes. Both visible (curled wing) and lethal phenotypes were observed (Table 1).

Identification of conditional, dominant mutations affecting life span: Thirteen *PdL* lines were tested for conditional, dominant effects specific to the aging period of the life cycle. These were the 13 lines where DOX feeding and gene overexpression during development was found to cause lethal or visible phenotypes. Each of the 13 lines (as well as Oregon-R wild-type controls) was crossed to *rtTA* in the absence of DOX, to generate 400 age-synchronized male flies containing both constructs. At 4 days of age the males were split into control and experimental groups, with experimentals placed on culture media supplemented with 250 μg/ml DOX. The flies were transferred to fresh vials every 2 days and the number dead was recorded. Mean life span was calculated, and the percentage difference between control and experimental groups is presented (Table 1). For several lines exhibiting a change in life span with DOX, the experiment was repeated and the results of both experiments are presented. Half of the lines exhibited a conditional, dominant phenotype of reduced life span, with decreases ranging from −4.7 to −32%. The high frequency of negative effects on life span observed is likely due to the fact that this set of lines was not random, but rather one where expression during development had been found to be disruptive or lethal. In contrast, line *PdL(3)19B3* exhibited a reproducible increase in life span of ~10%, while the control of Oregon-R wild type crossed to *rtTA* gave no significant change in life span.

Molecular characterization of *PdL* mutations: Southern analysis was used to determine the copy number of new *PdL* inserts in each line (data not shown; summarized in Table 1). Three lines containing single inserts were chosen for molecular analysis. For each of these lines excision of the *PdL* insert by crossing to Δ2-3 transposase source reverted the mutation. Chromosomal regions flanking the 3′ end of the insert were amplified by inverse PCR and sequenced. Comparison of the flanking sequences with the Drosophila genome database allowed mapping of the site of *PdL* insertion and the identification of the mutated gene. Line *PdL(3)19B3* (rough eye/increased life span) contained an insert in the 5′-UTR of the α-*mannosidase II* (*dGMII*) gene, 67 bp 5′ of the ATG translation start codon (Figure 3A). α-Mannosidase II is a Golgi apparatus enzyme involved in protein glycosylation (RABOUILLE *et al.* 1999). RNA was isolated from flies containing the *PdL(3)19B3* insert and *rtTA*, cultured ±DOX. Northern analysis revealed a 4-kb transcript that hybridized to a probe derived from the *dGMII* exon 4 and that was induced 15-fold by DOX (Figure 3D). As expected, the DOX-induced transcript is slightly larger than the endogenous *dGMII* transcript. This is because *PdL* is inserted near +1 of *dGMII*, and *PdL* contributes an extra 86 bp of 5′-UTR sequences.

It was of interest to determine if overexpression was limited to the *dGMII* gene or whether some DOX-induced transcription might read through to adjacent downstream gene(s). The Northern blot was hybridized

TABLE 1

PdL mutations and controls

Line name	No. of insertions (gene mutated)	Developmental phenotype	Penetrance[a] (%)	Life-span phenotype[b] −DOX	Life-span phenotype[b] +DOX	Life-span phenotype[b] % change (*P*)	Recessive phenotype	Comments
PdL(3)3G	2 (*pumilio*)	Curled wing	91	44.30 ± 0.515	43.72 ± 0.490	−1.3 (0.416)	Curled wing	Allelic to *PdL(3)3B2*
PdL(3)3B2	1 (*pumilio*)	Curled wing	67	44.73 ± 0.858	44.53 ± 0.966	−0.4 (0.878)	Curled wing	Allelic to *PdL(3)3G, reverted*
PdL(3)58A2/TM3	3 (*pumilio*)	Curled wing	95	65.45 ± 0.852	62.34 ± 0.968	−4.7 (0.0176)	WT	Complements *PdL(3)3G*
PdL(3)19B3/TM3	1 (*dGMII*)	Rough eye	100	50.98 ± 0.763	54.74 ± 0.835	+7.55 (0.001)	Viable	*reverted*
				56.47 ± 1.113	63.47 ± 1.003	+12.4 (<0.0001)		
PdL(3)35B1/TM3	4	Blistered wing	100	57.25 ± 0.888	53.80 ± 0.894	−6.0 (0.0065)	Lethal	
PdL(3)11A3/TM3	1 (*ash1*)	Lethal	100	49.91 ± 0.765	44.25 ± 0.566	−11 (<0.0001)	Lethal	*reverted*
				44.70 ± 0.613	38.67 ± 0.653	−14 (<0.0001)		
PdL(3)3A/TM3	2	Lethal	100	50.10 ± 0.881	49.56 ± 0.877	−1.1 (0.662)	Lethal	
PdL(3)2B1	3	Lethal	100	52.64 ± 1.216	38.46 ± 0.696	−27 (<0.0001)	Viable	
				57.49 ± 1.324	40.73 ± 0.930	−29 (<0.0001)		
PdL(3)43C1/TM3	3	Lethal	100	61.34 ± 0.951	56.37 ± 0.906	−8.1 (0.0002)	Lethal	
PdL(3)72A3/TM3	3	Lethal	96	77.65 ± 1.059	76.13 ± 0.884	−2.0 (0.268)	Lethal	
PdL(3)73A1/TM3	3	Lethal	98	54.53 ± 0.937	57.60 ± 0.936	+5.7 (0.0206)	Lethal	
PdL(3)75A3/TM3	3	Lethal	100	70.36 ± 1.639	71.41 ± 1.474	+1.5 (0.631)	Lethal	
PdL(2)14B1 Sp/SM5	1	Lethal	100	45.50 ± 1.178	36.06 ± 0.969	−21 (<.0001)	Lethal	
				47.05 ± 1.159	32.02 ± 0.809	−32 (<0.0001)		
PdL(2)45C1 Sp/SM5	3	Lethal	92	ND	ND	ND	Lethal	
Oregon-R				76.14 ± 1.498	74.45 ± 1.492	−2.2 (0.424)		
				71.27 ± 1.407	72.46 ± 1.247	+1.6 (0.528)		

WT, wild type; ND, not done.

[a] Penetrance calculations: For visible mutations penetrance = 100* [mutant/(WT + mutant)]; for lethal mutations penetrance = 100* [1 − (no. of escapers/no. of expected)].

[b] Mean life span in days at 25°, ±SEM, *P* value for unpaired, two-sided *t*-test.

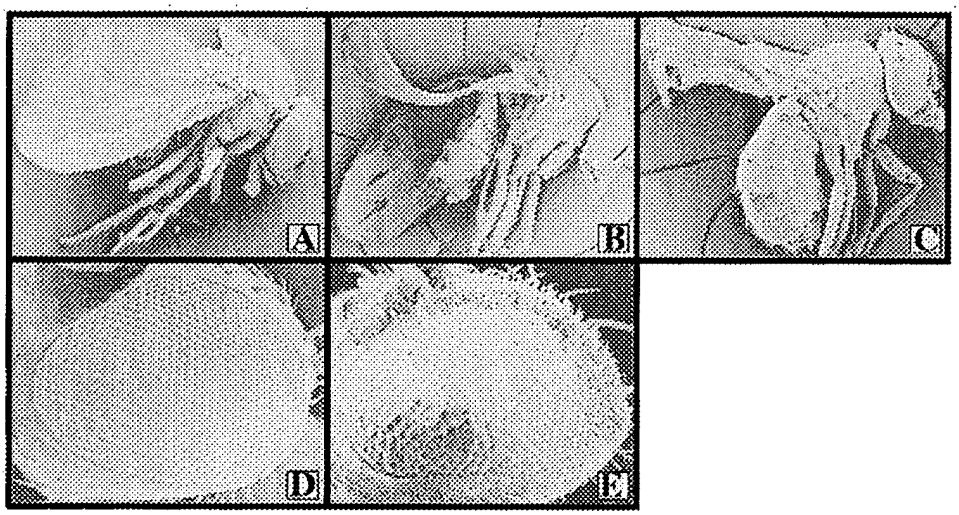

FIGURE 2.—Phenotypes of conditional, dominant mutations affecting development. Selected control and DOX-cultured flies were examined by scanning electron microscopy. (A) Control flies containing *PdL* insert 3B2 in the *pumilio* gene and the *rtTA* transactivator, genotype *w;PdL(3)3B2/rtTA(3)E2*. (B) Experimental flies containing *PdL* insert 3B2 in the *pumilio* gene and the *rtTA* transactivator, genotype *w;PdL(3)3B2/rtTA(3)E2*. The flies were cultured throughout larval and pupal development in the presence of 250 μg/ml DOX, in parallel with the control flies in A. (C) Experimental flies containing *PdL* inserts and the *rtTA* transactivator, genotype *w;PdL(3)35B1/rtTA(3)E2*, cultured throughout larval and pupal development in the presence of 250 μg/ml DOX. Control flies cultured without DOX were wild type and were identical to those in A (data not shown). (D) Compound eye of control flies containing *PdL* insert 19B3 in the *dGMII* gene and the *rtTA* transactivator, genotype *w;PdL(3)19B3/rtTA(3)E2*. (E) Compound eye of experimental flies containing *PdL* insert 19B3 in the *dGMII* gene and the *rtTA* transactivator, genotype *w;PdL(3)19B3/rtTA(3)E2*. The flies were cultured throughout larval and pupal development in the presence of 250 μg/ml DOX, in parallel with the control flies in D.

to a probe specific for *CG16765*, which is the next downstream ORF predicted by the Drosophila genome sequence (Figure 3A). The probe hybridized only to a very faint band of approximately the size predicted for the *CG16765* transcript (2.2 kb) that was not detectably altered by DOX (Figure 3D and additional data not shown). A probe specific for the intergenic region gave no detectable hybridization (data not shown). While this does not rule out the possibility that a small amount of transcription might read through the *dGMII* gene, any such transcription that might be occurring does not appear to result in detectable stable RNA.

Line *PdL(3)11A3* (lethal/decreased life span) contained an insert in the *absent, small or homeotic discs 1* (*ash1*) gene, 206 bp 5′ of the normal *ash1* transcription initiation site (Figure 3B). *ash1* is a member of the trithorax group of genes and encodes an RNA polymerase II transcription factor that positively regulates expression of homeotic genes during development (LAJEUNESSE and SHEARN 1995; TRIPOULAS *et al.* 1996). RNA was isolated from flies containing the *PdL(3)11A3* insert and *rtTA*, cultured ±DOX. Northern analysis revealed an 8.1-kb transcript that hybridized to a probe derived from the *ash1* exon 1 and that was induced fourfold by DOX (Figure 3E). For both the *dGMII* and *ash1* mutations, the size of the DOX-induced transcripts suggests that they are correctly terminated and processed.

Line *PdL(3)3B2* (curled wing/unchanged life span) contained an insert near the middle of the 120-kb intron 8 of the *pumilio* gene (Figure 3C). This intron is one of the largest known in Drosophila and contains an ~500-bp "hot spot" for *P*-element insertion (PARISI and LIN 1999). Pumilio is an RNA binding protein involved in regulation of translation and is required both maternally and zygotically for multiple stages of development (FORBES and LEHMANN 1998; WHARTON *et al.* 1998). Two other *PdL* mutant lines exhibited a similar curled wing phenotype and were found to have insertions at the same location within *pumilio* (Figure 3C). Northern analysis of these lines revealed a complex pattern of endogenous and DOX-induced *pumilio* transcripts common to each of the three lines (Figure 3F). A probe derived from *pumilio* exons 9 + 10 hybridized to the native 6.9-kb *pumilio* RNA and a 0.4-kb RNA. A probe derived from *pumilio* intron 8 revealed that part of the signal at 6.9 kb results from a second RNA originating just downstream of the *P*-element hotspot. This transcript begins in the intron, was rare or absent in adult males, and was induced 3- to 10-fold by DOX. Two smaller RNAs were also strongly induced by DOX. Searches of the Drosophila cDNA database with sequences from this region of *pumilio* intron 8 identified several cDNAs derived from wild-type Drosophila tissue culture cells (diagrammed in Figure 3C). These cDNAs also initiate just downstream of the hot spot, contain an ATG start codon in an alternative exon spliced in frame to *pumilio* exon 9, and encode a potential alternative pumilio protein. Taken together, the data suggest that in the three mutant lines examined, *PdL* has inserted at the 5′ end of a previously unidentified *pumilio* internal promoter and activated expression of alternative *pumilio* gene product(s).

DISCUSSION

PdL was found to generate conditional, dominant mutations at high frequency. Approximately 7% of mu-

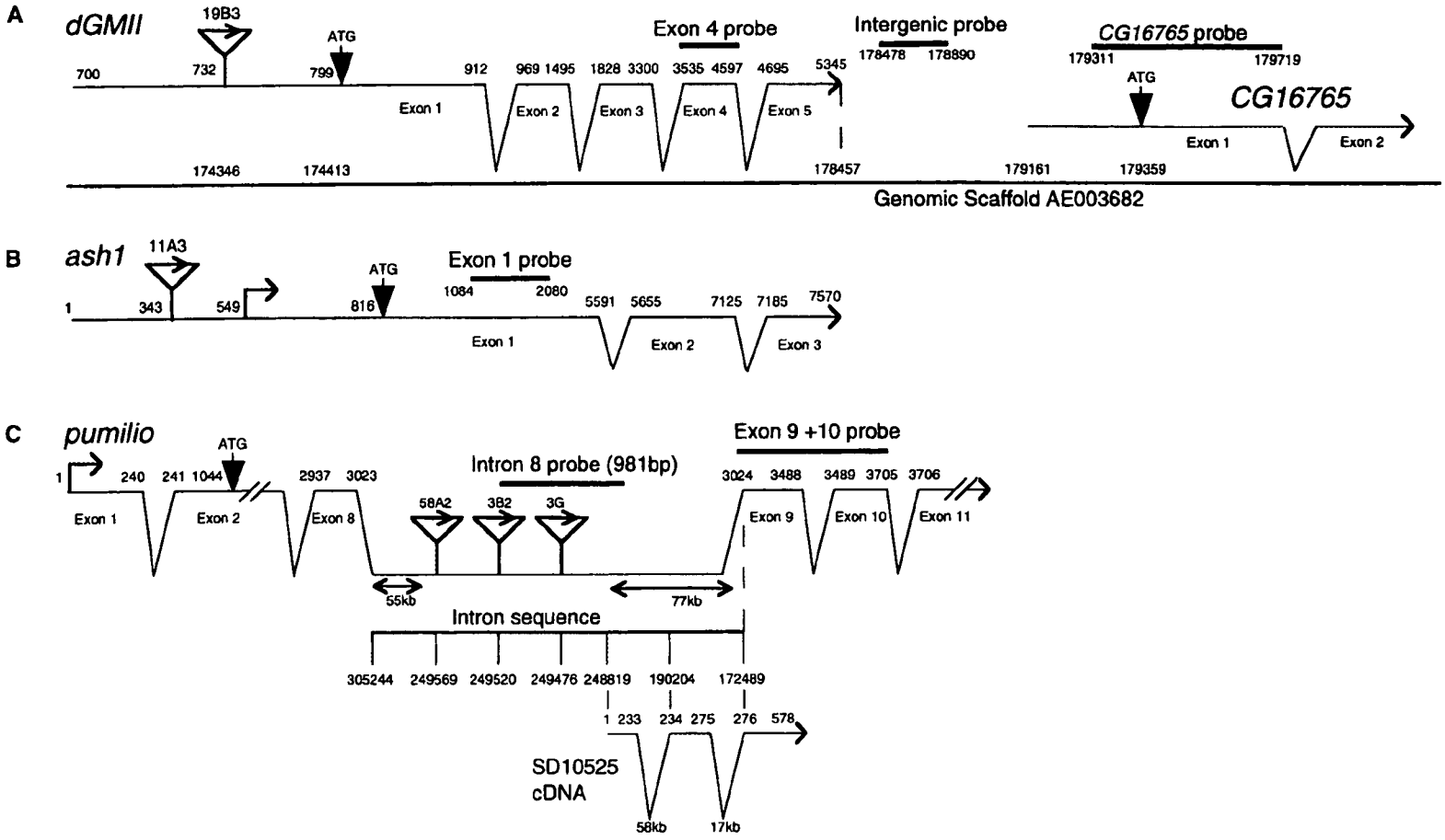

FIGURE 3.—Molecular characterization of selected mutations. (A–C) Intron and exon boundaries of the mutated genes are indicated, with numbering according to DNA sequences obtained from NCBI web page (http://www.ncbi.nlm.nih.gov/). Locations for transcriptional initiation and ATG translation start codons are indicated by arrows. *PdL* inserts are indicated by triangles and each is oriented 5′ to 3′, as indicated by internal arrows. DNA fragments used as probes in Northern analyses are indicated above each diagram. (A) *dGMII* [sequence accession no. AJ132715; genomic scaffold sequence AE003682 (ADAMS *et al.* 2000)]. (B) *ash1* (sequence accession no. U49439). (C) *pumilio* (sequence accession no. L07943; intron sequence and numbering are from Drosophila genomic scaffold sequence AC no. AE003681; SD10525 cDNA sequence is from AC no. AI543655). (D–F) Northern analysis of selected lines. Oregon-R wild-type strain and the indicated *PdL* mutant strains were crossed to *rtTA*. Total RNA was isolated from progeny cultured ±DOX, transferred to Northern blots, and hybridized with the gene-specific probes indicated (A–C). Ribosomal protein 49 gene *Rp49* was used as control for loading. Two amounts of RNA were loaded for each sample (1× and 2×, as indicated), and signals were quantitated by phosphoimager. (D) *dGMII* mutant strains and controls. (E) *ash1* mutant strain and controls. (F) *pumilio* mutant strains and controls.

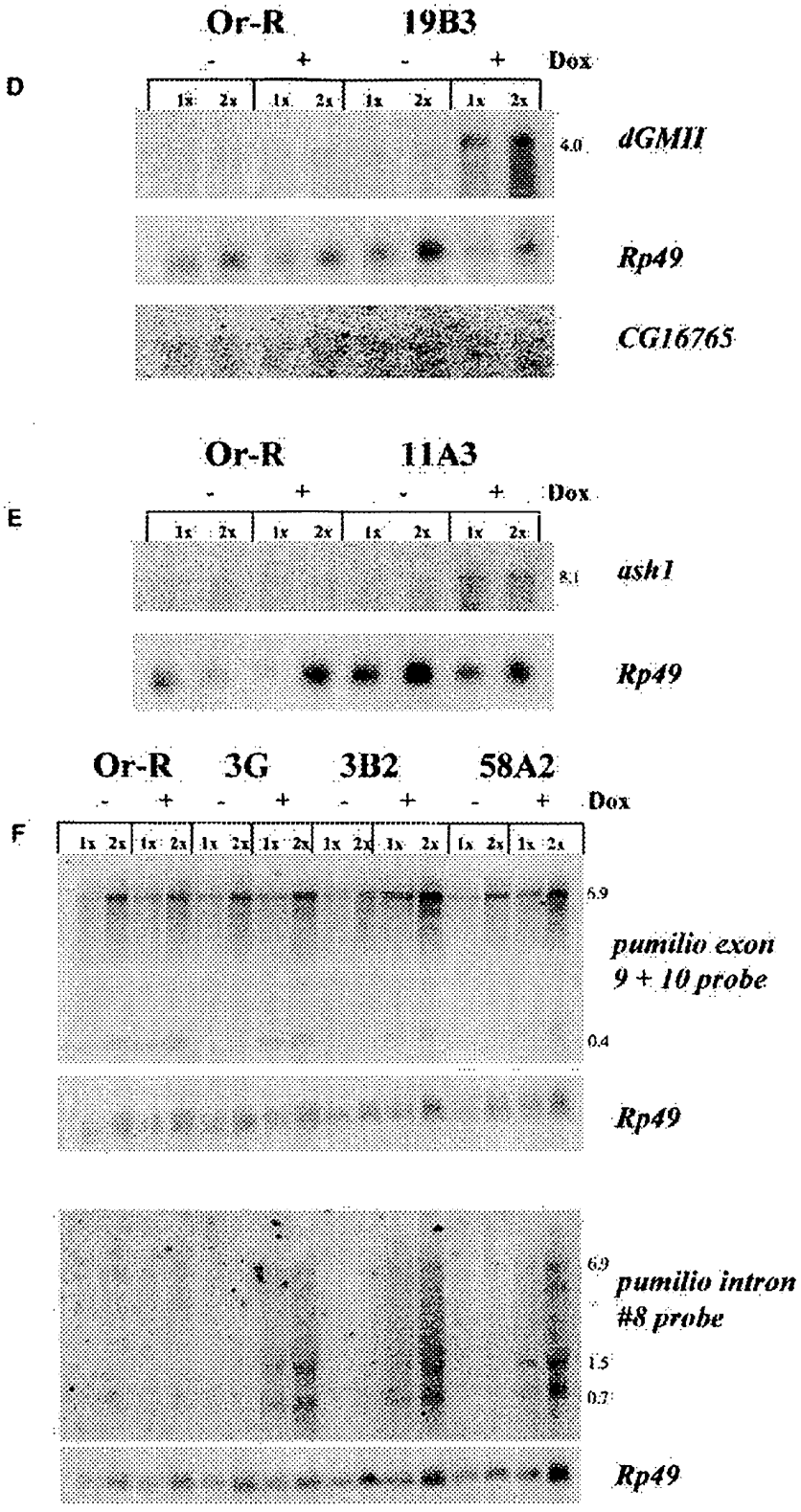

FIGURE 3.—Continued.

tated chromosomes yielded visible or lethal phenotypes when *PdL* was activated by DOX feeding during larval and pupal development. It is likely that the frequency at which *PdL* inserts cause gene overexpression is significantly higher than the frequency at which mutations were identified in these experiments. Genes affecting

embryogenesis would not have been detected because DOX is not present inside the eggshell. If desired, such genes could potentially be detected by prefeeding the mothers DOX. In addition, not all overexpressed genes are expected to produce lethal or obvious visible phenotypes, and therefore overexpression of such genes would not have been detected.

In most of the mutant lines, the mutated chromosome had multiple *PdL* insertions, and there are likely two reasons for this result. First, the starting insert line selected for mobilization had a relatively high transposition frequency, which reduces the effort required to identify new insertions but favors multiple inserts. Second, chromosomes with multiple inserts will be more likely to have a new mutation. The chromosomes examined were ones selected for mutant phenotype, and this selection may have enriched for multiple insert chromosomes.

The temporal control of gene overexpression and mutant phenotype provided by *PdL* extends the usefulness of *P*-element mutagenesis. The conditional nature of the *PdL* mutations allowed identification of stage-specific gene misexpression phenotypes that it had not previously been possible to study. For example, the *PdL* insertions in *pumilio* produced a novel curled wing phenotype and suggested the existence of a novel *pumilio* internal promoter. Misexpression of *ash1* specifically in the adult revealed a previously unknown negative effect on life span. Finally, the *PdL* insertion in *dGMII* yielded some of the first mutant phenotypes described for this gene. Feeding of DOX during larval and pupal development disrupted eye development, while feeding of DOX only during adulthood resulted in an ~10% increase in life span. α-Mannosidase II is involved in protein glycosylation and the likely mechanism for either phenotype in Drosophila is currently unclear. In mouse, mutation of the homologous gene encoding α-mannosidase II causes a systemic autoimmune disease that becomes more severe with increasing age, and that resembles human lupus erythematosus (Chui *et al.* 2001). Very recently another group has used a *P*-element "gene-trap" method to generate a lethal insertion in the Drosophila *dGMII* gene (Lucasovich *et al.* 2001).

In previous studies, *P* elements with outwardly directed promoters generated (nonconditional) mutations at frequencies ranging from 2 to 64%, depending on the particular promoters used to drive expression (Rorth 1996; Rorth *et al.* 1998; Toba *et al.* 1999). In those studies the elegant GAL4/UAS system (Brand and Perrimon 1993) was used to provide tissue-specific gene misexpression and tissue-specific phenotypes resulting from a promoter directed out of the end of an engineered *P* element, called "EP." Activation of EP line gene misexpression in larval imaginal tissues was often associated with wing and eye phenotypes similar to those observed here for *PdL*. The large *pumilio* intron 8 is a hot spot for *P*-element insertion (Parisi and Lin 1999); however, no mutant phenotypes were observed for multiple EP insertions at this site (Rorth *et al.* 1998; P. Rorth, personal communication). Because of its unique temporal regulation, *PdL* mutagenesis is expected to identify gene functions not detected with other methods.

The conditional mutations caused by *PdL* are dominant gain-of-function mutations, as opposed to the more common loss-of-function mutations resulting from gene disruptions. The gain-of-function phenotypes could result from expression of the gene in the wrong place or in the wrong amount, and both situations have been observed with EP mutagenesis (Rorth *et al.* 1998). An important consideration is that inappropriate expression of a protein by *PdL* might disrupt development or life span due to a nonspecific effect, such as by preventing cell differentiation or causing a novel, nonspecific toxicity. One way to confirm that the gene is involved in a particular process or pathway of interest is to also examine the loss-of-function phenotype for the gene. This is facilitated by the fact that the *PdL* insertion may be located such that it disrupts the gene and thereby generates a loss-of-function phenotype when made homozygous. This appears to be the situation with lines *PdL(3)3G* and *PdL(3)3B2* where both the overexpression and recessive phenotypes were a curled wing, and in nine *PdL* lines where the recessive phenotype was lethality. If a *PdL* insertion is located such that it does not disrupt the gene, such disruptions can often be generated by imprecise excision or local transposition of the element (Salz *et al.* 1987; Tower *et al.* 1993; Spradling *et al.* 1995). The dominant and conditional nature of *PdL* mutations should therefore allow for the relatively rapid and efficient identification of genes of potential interest, which can then be confirmed to be involved in the relevant process or pathway by more traditional analyses. This general strategy has been shown to be highly productive in studies of Drosophila development using EP misexpression mutagenesis (Rorth *et al.* 1998, 2000; Huang and Rubin 2000; Mata *et al.* 2000).

The conditional nature of the mutations generated by *PdL* makes them particularly well suited to studies of aging and life span. The temporal control of gene overexpression allows the investigator to avoid any toxic or confounding effects during development and study gene functions specifically in the adult. In addition, like other quantitative traits, life span is greatly affected by genetic background, and *PdL* provides powerful controls for this variable. Control and mutant (overexpressing) flies have identical genetic backgrounds, and therefore any differences observed must be due to DOX and the subsequent gene overexpression or misexpression. Several lines were identified where activation of *PdL* in the adult caused reductions in life span, with decreases up to −32%. This high frequency of negative effects was not surprising, given that these were lines where activation of *PdL* during development was disruptive or lethal.

Increased life span is expected to be a more rare phenotype of gene misexpression. A small but reproducible increase in life span of ~10% was associated with the *PdL* insertion in the *dGMII* gene; however, it is not possible to predict how common this phenotype is likely to be based on this one example. It is known that induced overexpression of at least two additional genes, Cu/ZnSOD and MnSOD, can extend adult Drosophila life span based on experiments using different gene expression systems (PARKES *et al.* 1998; SUN and TOWER 1999; J. SUN, D. FOLK, T. J. BRADLEY and J. TOWER, unpublished data). It seems likely that additional genes will exist that can extend life span when overexpressed in the adult. The potential for *PdL* mutagenesis to test large numbers of genes should provide an efficient way to identify additional genes that regulate aging and life span.

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Research

Doxycycline-induced expression of sense and inverted-repeat constructs modulates *phosphogluconate mutase (Pgm)* gene expression in adult *Drosophila melanogaster*

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Abstract

Background: A tetracycline-regulated (conditional) system for RNA interference (RNAi) would have many practical applications. Such a strategy was developed using RNAi of the gene for *phosphogluconate mutase* (*Pgm*). *Pgm* is a candidate lifespan regulator: Pgm^S allele frequency is increased by selection for increased lifespan, whereas Pgm^M and Pgm^F allele frequencies are decreased.

Results: The *Pgm* alleles were cloned and sequenced and were found to differ by amino-acid substitutions consistent with the relative electrophoretic mobilities of the proteins. The 'tet-on' doxycycline-regulated promoter system was used to overexpress Pgm^S in a wild-type (Pgm^M) background. Enzyme activity increases of two- to five-fold were observed in five independent transgenic lines. Tet-on was also used to drive expression of an inverted-repeat fragment of *Pgm* coding region. The inverted-repeat transcript was expected to form a dsRNA hairpin, induce RNAi, and thereby reduce endogenous *Pgm* gene expression at the RNA level. Endogenous *Pgm* RNA levels in adult flies were found to be reduced or eliminated by doxycycline treatment in five independent inverted-repeat transgenic lines. Our results show that doxycycline-regulated expression of inverted-repeat constructs can cause a conditional reduction in specific gene expression. The effect of sense and inverted-repeat construct expression on lifespan was assayed in multiple transgenic lines. Under the conditions tested, altered *Pgm* gene expression had no detectable effect on adult *Drosophila* lifespan.

Conclusions: A system for conditional RNAi in *Drosophila* adults shows promise for assay of gene functions during aging. Our results indicate that *Pgm* does not have a simple strong effect on longevity.

Background

Conditional gene expression systems such as promoters regulated by tetracycline or doxycycline (DOX) have several advantages for testing the effects of genes on aging and lifespan [1-3]. In the 'tet-on' system, feeding DOX to *Drosophila melanogaster* causes high levels of transgene expression in all tissues. By waiting until the young adult stage to administer DOX, all of pre-adult development is identical between control and experimental groups, and any difference in lifespan must be due to changes in the adult. Subtle differences in the genetic background of *Drosophila* strains can have significant effects on lifespan

[4-6]. With the tet-on system, control and experimental animals have identical genetic backgrounds, and therefore any differences in lifespan must be due to DOX administration and transgene expression. DOX itself, and overexpression of control genes such as *Escherichia coli lacZ*, have no detectable effects on lifespan [1]. In contrast, several genes have been identified for which DOX-regulated overexpression has negative effects on lifespan, and overexpression of the *dGMII* gene, encoding α-mannosidase II, was associated with slightly increased lifespan [7].

Expression of antisense RNA has long been known to be able to inhibit gene expression in *Drosophila* and other organisms [8-12]. Double-strand RNA (dsRNA) formed by hybridization of sense and antisense sequences is thought to initiate a pathway in which homologous RNA sequences are destroyed. This phenomenon has been referred to as post transcriptional gene silencing (PTGS) or RNA interference (RNAi). Expression of inverted-repeat constructs, where the transcript is expected to fold into a dsRNA hairpin, has been shown to be an efficient initiator of RNAi [13-15]. In experiments reported here, the tet-on system was used to drive expression of inverted-repeat constructs in *Drosophila* to determine if a conditional system for RNAi could be created.

In natural populations, lifespan and reproductive period are thought to co-evolve [16]. The duration of the reproductive period is subject to natural selection, and lifespan is altered as a consequence. Significant experimental support for this model has come from laboratory selection experiments with *D. melanogaster* [17-21]. Genetically heterogeneous *Drosophila* populations have been selected over hundreds of generations for late-life reproduction. Such selection results in strains with increased lifespan relative to controls. Selection is thought to function by altering the frequency of gene alleles present in the starting population. Selected strains exhibit a number of correlated phenotypes. The correlated phenotypes can vary somewhat depending on the starting strains used; however, increased stress-resistance appears to be common to all or most long-lived strains [22-26]. In one well studied set of five replicate control (B) and long-lived (O) strains, lifespan was doubled and was correlated with increased stress-resistance and increased glycogen and lipid stores [27,28].

Starch-gel electrophoresis has been used to assay for changes in the frequency of enzyme electrophoretic alleles in the O and B lines. A change in the frequency of Cu/Zn-superoxide dismutase (Cu/ZnSOD) alleles was observed, with the more active allele enriched in the long-lived O strains [29]. Consistent with this observation, overexpression of Cu/ZnSOD in transgenic *Drosophila* has been shown to be sufficient to cause significant increases in lifespan [30,31]. The most dramatic change in allele frequency in the O and B lines was observed for the gene for *phosphogluconate mutase* (*Pgm*) [32]. *Pgm* exists in three electrophoretic forms: fast (Pgm^F), medium (Pgm^M) and slow (Pgm^S). Pgm^M predominates in the B strains, whereas Pgm^S allele frequency is on average tenfold higher in the long-lived O strains relative to the B controls. When O strains were taken off selection for several generations (back-selection), lifespan decreased and Pgm^S allele frequency was reduced to levels more like those in B strains. These results suggest that *Pgm* or a closely linked gene is responding to selection, and make Pgm^S a candidate lifespan regulator.

Pgm encodes the enzyme phosphoglucomutase, which interconverts glucose 1-phosphate and glucose 6-phosphate. Its activity is therefore important for both glycolysis and glycogen synthesis, and the altered *Pgm* allele frequency might therefore be relevant to the increased glycogen and lipid stores of the O strains. If the increased Pgm^S allele frequency is contributing to the unique phenotypes of the long-lived O strains, this might be because Pgm^S has increased or decreased enzyme activity, or enzyme activity has been altered in some way (perhaps by a change in its regulation). One way to begin to test these models experimentally is to engineer transgenic flies with increased or decreased *Pgm* expression and assay for effects on lifespan.

Results

Multiple strains were generated that were homozygous for each of the F, M and S electrophoretic alleles of *Pgm*, by appropriate crosses to a third chromosome balancer stock. *Pgm* enzyme activity and lifespan varied greatly across the strains with no correlation with *Pgm* allele (data not shown). This result was expected, as differences in genetic background between such purified chromosome strains has profound effects on lifespan and the activities of various enzymes [5,6,33]. The *Pgm* coding-region sequences were cloned and sequenced from the Pgm^F, Pgm^M and Pgm^S homozygous strains and from the Canton-S wild-type strain. Amino-acid substitutions were identified that predicted pI values for the alleles that correlated with their mobility on starch gels (Figure 1). The substitution of T for A in Pgm^S creates the amino-acid sequence TTK (in the single-letter amino-acid code) which is a potential phosphorylation site for protein kinase C (PKC) that is absent in Pgm^M and Pgm^F.

If Pgm^S contributes to the increased lifespan of the O lines, this might be due to increased enzyme activity, decreased enzyme activity, or alteration in enzyme activity in some other way such as in its regulation or subcellular localization. The tet-on system was used to test whether simply increasing or decreasing *Pgm* expression would be sufficient to alter lifespan. A P-element transformation vector called USC1.0 was generated, which had unique *Eco*RI and *Pst*I sites downstream of the tet-on doxycycline-regulated promoter (Figure 2a). USC1.0 also contains the mini-*white*$^+$

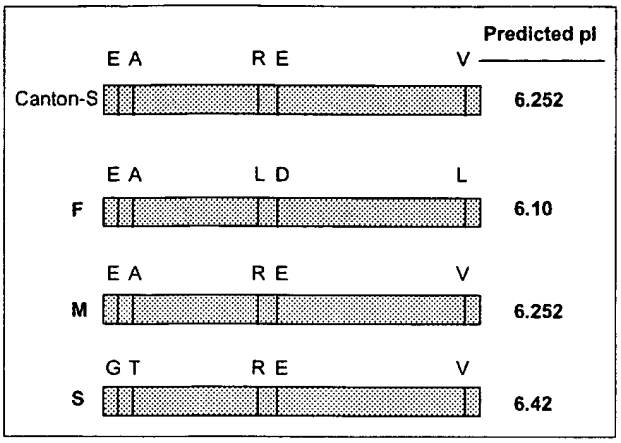

Figure 1
Sequence alterations in various *Pgm* electrophoretic alleles. The amino acids that differ between the Pgm^F, Pgm^M, Pgm^S and wild-type Canton-S alleles are indicated in single-letter code, with the predicted pI values at the right. The GenBank accession numbers for the sequences are AF416982, AF416981, AF416983 and AF416984, respectively.

transformation marker gene. The Pgm^S cDNA sequences were cloned into USC1.0 to create the PGMsense construct (Figure 2b), which should allow overexpression of the PGM^S enzyme. A fragment of Pgm^S coding region of approximately 1 kilobase (kb) (from +711 to +1,794) was cloned into USC1.0 in an inverted-repeat orientation to create construct PGMinvrpt (Figure 2c), which should potentially cause conditional RNAi. For each PGM construct five independent transgenic lines were generated using standard methods. As a control, two trangenic lines were generated containing the USC1.0 vector in which no transgene is expressed.

Expression of the transgenic constructs and the endogenous *Pgm* gene was assayed by northern blot, with or without DOX (Figure 3a). The *Pgm* inverted-repeat sequences were used as probes and should hybridize to both transgenes and the endogenous *Pgm* transcript. In the sense-construct lines, feeding DOX resulted in efficient expression of the transgene at levels five- to ten-fold greater than the endogenous transcript. DOX-induced expression of the inverted-repeat construct had two effects detected by northern blot. First, DOX produced a smear of hybridization resulting from the inverted-repeat transcript (Figure 3a). The smear pattern may have resulted from incomplete denaturation of the hairpin structure and/or instability of the inverted-repeat transcript. Second, the level of the endogenous *Pgm* transcript was found to be reduced by DOX feeding.

The northern blots were also hybridized with a *Pgm*-specific probe corresponding to *Pgm* coding-region sequences (+1 to +700) located outside the region used to create the inverted repeat. This probe is therefore specific for the endogenous *Pgm* transcript. Using this probe the smear of hybridization derived from the inverted-repeat construct was no longer detected, and the endogenous *Pgm* RNA levels were more readily observed and quantitated (Figure 3b). DOX induced expression of the inverted-repeat construct was found to cause a reduction of endogenous *Pgm* RNA levels. The decrease varied across the five transgenic lines, with reductions ranging from 1.3- to 24-fold. The inverted-repeat construct therefore appears to function as expected to induce RNAi and cause decreased expression of the endogenous *Pgm* gene. In the control strains where no transgene is expressed, DOX administration was found to have no effect on *Pgm* transcript levels (Figure 3c).

The indirect PGM activity assay was used to assay for changes in *Pgm* gene expression at the protein level. The sense construct yielded conditional overexpression of PGM activity in each transgenic line, with increases ranging from two- to five-fold (Table 1). Potential reductions in PGM activity were more difficult to assay because of the inherent limitations of the assay. The PGM activity assay is indirect and is coupled to the conversion of $NADP^+$ to NADPH. The extract will contain other activities capable of converting $NADP^+$ to NADPH, thereby creating a significant background activity. Decreases in PGM activity could not be reliably measured because of this unavoidable background. The PGMinvrpt construct yielded decreases in PGM activity of as much as 50%; however, the effect was quite variable from experiment to experiment (Table 1). In the control strains where no transgene is expressed, DOX administration was found to have no consistent effect on PGM levels (Table 1).

To determine whether altered *Pgm* gene expression could affect lifespan, mean lifespan was assayed in multiple PGMsense and PGMinvrpt transgenic lines with or without DOX treatment. The percentage change in mean lifespan caused by DOX and transgene expression is presented for each line. Lifespan was observed to vary across transgenic lines and replicate experiments with changes ranging from -7% to +6%, with one outlier at +16% (Table 2). However, no change in lifespan was observed that was consistent across the sense or inverted-repeat lines or in multiple experiments. The lifespan assays were repeated at 29°C, and again no consistent changes were observed (Table 3). We conclude that under these conditions, altered *Pgm* expression does not have a detectable effect on adult *Drosophila* lifespan.

Discussion

The phenomenon of RNAi (or PTGS) is of great interest for two reasons [9-12]. First, it represents an evolutionarily conserved pathway that probably has important functions in the regulation of gene expression and the control of transposable elements. Second, it provides a means for researchers to test gene functions by experimentally downregulating

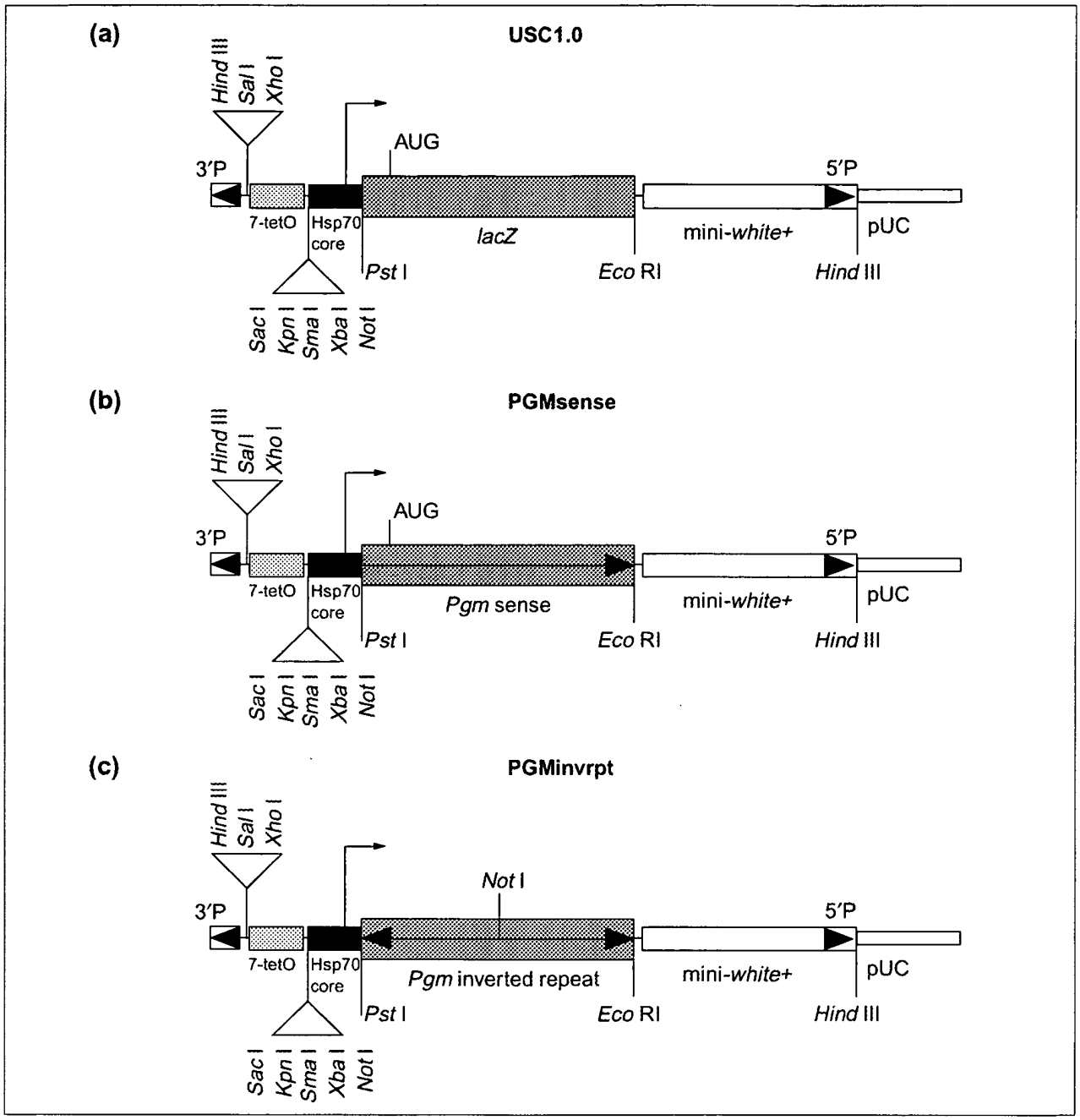

Figure 2
P-element transformation constructs. **(a)** The USC1.0 vector for the tet-on system. Unique *Pst*I and *Eco*RI sites are located downstream of the tet-on promoter, enabling cloning of cDNAs. cDNAs should contain their own ATG start codons and polyadenylation signal sequences. **(b)** The PGMsense construct for conditional overexpression of Pgm^S. **(c)** The PGMinvrpt construct, containing an approximately 1 kb inverted repeat of Pgm^M coding region, as indicated by inverted arrows.

expression of specific genes under well controlled conditions. In *Drosophila*, RNAi can be initiated by injection of dsRNA into embryos, and this has allowed identification of novel phenotypes for genes during development [34]. However, injection has limited potential application in the adult, as it is unlikely that the RNAi could be targeted to all tissues, or to specific tissues, and the trauma of injection is likely to have negative effects on phenotypes such as lifespan.

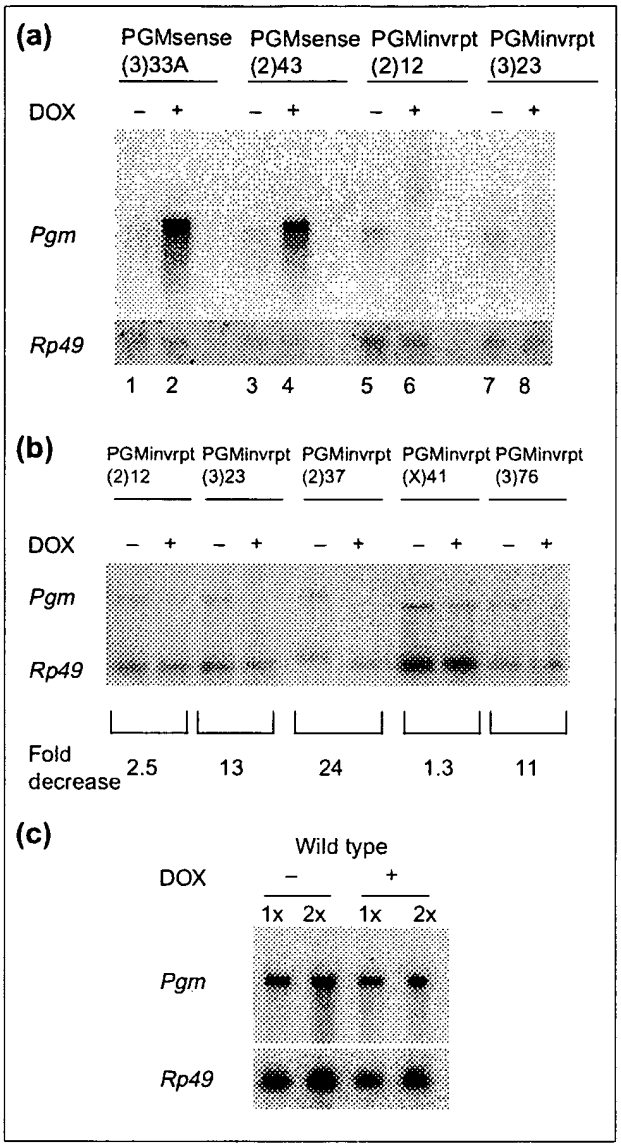

Figure 3
Northern analysis of PGMsense and PGMinvrpt construct lines. Total RNA was isolated from adult males of the indicated PGMsense lines, PGMinvrpt lines, and control line, with or without DOX treatment. **(a)** Northern blot hybridized with a probe that will recognize both endogenous and transgenic *Pgm* transcripts and with a probe specific for *Rp49* as a loading control. **(b)** Northern blot hybridized with a probe that will recognize only the endogenous *Pgm* transcript and with a probe specific for *Rp49* as a loading control. The fold decrease in *Pgm* transcript abundance is indicated below the lanes. **(c)** Northern blot of control strain hybridized with a probe that will recognize the endogenous *Pgm* transcript and with a probe specific for *Rp49* as a loading control.

Transgenic constructs have been used to cause RNAi in *Drosophila*, by using various promoters to drive expression of inverted-repeat fragments of gene coding regions [14,15]. Using the conditional tet-on promoter system to drive expression of an inverted-repeat provided conditional RNAi of the *Pgm* gene. This conditional system should have many applications, both in the study of the RNAi pathway itself and in the design of experiments where RNAi is used as a research tool. Conditional RNAi should be particularly useful to study phenotypes in the adult such as aging and lifespan. Conditional gene-expression systems have previously been used to identify positive regulators of lifespan, such as *Cu/ZnSOD* and *dGMII* [7,31]. In addition, mutations have been used to identify several genes in *Drosophila* that act as negative regulators of lifespan, such as *mth*, *INDY*, *InR* and *chico* [35-38]. For *mth* and *InR* the null phenotype of the genes is lethality. Therefore it is only rare hypomorphic alleles or allele combinations that allow the animals to survive to adult stage where increased lifespan can be observed. Conditional RNAi should provide a powerful means to study these genes and other negative regulators of aging and lifespan. Inactivation or downregulation of the genes specifically in the adult using tet-on RNAi may allow increased lifespan without confounding effects on development. Lam and Thummel have recently reported the use of a heat-shock gene promoter to drive expression of dsRNA, and the efficient conditional inhibition of gene expression during larval and pupal stages of *Drosophila* development [39].

Genetic selection of *Drosophila* populations for late-life reproduction caused the correlated phenotype of increased lifespan and increased the frequency of the slow electrophoretic allele, Pgm^S, of the *Pgm* gene. If Pgm^S contributes to the increased lifespan phenotype of the selected strains, it might be because Pgm^S has increased activity, decreased activity or activity that is altered in some way such as through its regulation or subcellular localization. The *Pgm* alleles were cloned and sequenced and were found to differ by amino-acid substitutions consistent with the relative electrophoretic mobilities of the encoded enzymes.

A recent study reported the sequencing of several fast, medium and slow electrophoretic alleles of *Pgm* from a wild population of *D. melanogaster* [40]. The sequences reported in that study (Verrelli alleles) are comparable to those reported here (Ives alleles) as follows: the medium Verrelli allele used for alignment is identical in amino-acid sequence to the Ives medium allele. The Verrelli fast alleles are identical to the Ives fast allele in each of the three amino-acid positions at which the Ives fast differs from the Ives medium (wild-type) allele. There are a number of differences between the Verrelli slow alleles and the Ives slow allele. The Verrelli slow alleles are not all identical to each other. The Ives slow allele differs from the Ives medium (wild-type) amino-acid sequence at two positions (amino-acid positions 6 (E → G) and 9 (A → T)). The amino-acid change in the Ives slow allele at position 6 (E → G) is not reflected in any of the Verrelli slow alleles. The second amino-acid change in the Ives slow allele (at position 9) is shared by two of the Verrelli slow alleles. In addition, the Verrelli slow alleles have

Table 1

PGM enzyme activity

		Enzyme activity (ΔOD/μg/min)		
Line name	Sex	-DOX	+DOX	Percentage change (*p*-value)
PGMsense(3)33A1	Male	0.1105 ± 0.014	0.5926 ± 0.089	+436% (0.0008)
	Male	0.1035 ± 0.004	0.5901 ± 0.070	+470% (0.0003)
PGMsense(3)33B1	Male	0.1018 ± 0.013	0.4585 ± 0.010	+350% (3.0E-06)
	Male	0.1138 ± 0.014	0.5829 ± 0.018	+412% (3.6E-06)
PGMsense(2)43A2	Male	0.0893 ± 0.010	0.4825 ± 0.014	+440% (2.3E-06)
	Male	0.1100 ± 0.020	0.6706 ± 0.085	+510% (3.6E-04)
PGMsense(2)52A1	Male	0.1131 ± 0.007	0.4939 ± 0.048	+337% (0.0002)
	Male	0.0925 ± 0.012	0.2608 ± 0.060	+182% (0.0088)
PGMsense(X)27A1	Female	0.1301 ± 0.003	0.3093 ± 0.047	+138% (0.0028)
PGMinvrpt(3)76	Male	0.1179 ± 0.0121	0.1162 ± 0.0069	-1.44% (0.83)
	Male	0.0966 ± 0.0076	0.0625 ± 0.0049	-35.3% (0.0028)
PGMinvrpt(3)23B1	Male	0.1198 ± 0.0117	0.0810 ± 0.0070	-32.4% (0.0078)
	Male	0.0789 ± 0.0081	0.0852 ± 0.0047	+7.98% (0.31)
PGMinvrpt(2)12A1	Male	0.1569 ± 0.0118	0.0723 ± 0.0077	-53.9% (0.0005)
	Male	0.0729 ± 0.0075	0.0736 ± 0.0041	+0.96% (0.89
PGMinvrpt(2)37A1	Male	0.2038 ± 0.0134	0.1241 ± 0.0077	-39.1% (0.0009)
	Male	0.1631 ± 0.0089	0.1179 ± 0.0054	-27.7% (0.0017)
PGMinvrpt(X)41A2	Female	0.2853 ± 0.014	0.1757 ± 0.002	-38.4% (0.0002)
Control 1	Male	0.1014 ± 0.0029	0.1019 ± 0.0093	+0.5% (0.932)
Control 2	Male	0.0997 ± 0.0038	0.1118 ± 0.0038	+12.0% (0.017)

amino-acid substitutions that are not shared by the Ives slow allele. The data are consistent with the conclusion that *Pgm* is highly polymorphic and subject to selection in natural populations [40,41]. Consistent with this idea, *Pgm* haplotypes and glycogen content have been found to vary among flies at different latitudes [42].

The tet-on promoter system allowed a test of the hypothesis that increasing or decreasing *Pgm* gene expression would affect lifespan. PGM enzyme levels were increased two- to fivefold using a sense transgenic construct, and *Pgm* RNA levels were decreased 1.3- to 24-fold by driving expression of an inverted-repeat construct. No changes in lifespan were detected that were consistent across multiple transgenic lines or replicate experiments. The results indicate that simply increasing or decreasing *Pgm* gene expression does not significantly affect the lifespan of adult *Drosophila*, at least under the conditions tested.

The Pgm^S allele used in these experiments was cloned from the Ives strain, which is the progenitor of the long-lived selected strains and their controls. The high degree of polymorphism of *Pgm* makes it possible that the slow electrophoretic allele in the Ives strain could consist of multiple DNA sequence forms. The failure to observe effects on lifespan could therefore conceivably have resulted from overexpression of the wrong DNA sequence form of Pgm^S. However, Pgm^S was cloned and sequenced from four independent lines derived from the Ives strain and the clones had the same DNA sequence. The data therefore suggest that most, if not all, of the Pgm^S alleles in these strains have the same sequence, making sequence heterogeneity an unlikely explanation of the present results.

The results suggest several possibilities for the relationship of *Pgm* allele frequency to the increased lifespan of the selected strains. The first is that Pgm^S might in fact contribute to the increased lifespan of the selected strains as a result of an increased or decreased activity, but that the effect on lifespan is too small to be detected in these assays. The lifespan assay appears to have a variability with control strains of approximately -5% to +5%, as seen in the data presented here and elsewhere (G.L., D. Bhole and J.T., unpublished results). It is therefore unlikely that an effect much smaller than 10% could be identified. The second possibility is that *Pgm* allele frequencies are altered because of selection for an unknown linked gene, and this possibility cannot be ruled out at this time. The experiments presented here

Table 2

Mean lifespan at 25°C

Line name	Sex	Mean lifespan (25°C) -DOX	Mean lifespan (25°C) +DOX	Percentage change (*p*-value)
PGMsense(3)33A1	Male	56.723 ± 0.763	56.048 ± 0.743	-1.19% (0.53)
	Male	66.921 ± 0.746	64.955 ± 0.731	-2.94% (0.060)
PGMsense(3)33B1	Male	58.167 ± 0.777	54.140 ± 0.817	-6.92% (0.0004)
	Male	68.169 ± 0.686	67.966 ± 0.614	-0.30% (0.83)
PGMsense(2)43A2	Male	68.256 ± 0.845	65.236 ± 0.870	-4.42% (0.013)
	Male	71.202 ± 0.730	67.268 ± 0.860	-5.53% (0.0005)
PGMsense(2)52A1	Male	63.720 ± 0.844	67.568 ± 0.939	+6.04% (0.0024)
	Male	70.610 ± 0.607	72.508 ± 0.803	+2.69% (0.060)
PGMsense(X)27A1	Female	89.660 ± 0.992	84.275 ± 0.806	-6.01% (<0.0001)
Control	Male	58.568 ± 0.939	58.930 ± 0.938	+0.62% (0.79)
PGMinvrpt(3)76	Male	59.957 ± 0.825	69.817 ± 0.547	+16.45% (<0.0001)
	Male	66.428 ± 0.561	66.801 ± 0.640	+0.56% (0.66)
PGMinvrpt(3)23B1	Male	55.415 ± 0.965	56.486 ± 1.014	+1.93% (0.45)
	Male	69.061 ± 1.302	71.339 ± 1.253	+3.30% (0.21)
PGMinvrpt(2)12A1	Male	71.111 ± 0.979	75.099 ± 1.100	+5.61% (0.0071)
	Male	75.958 ± 0.877	72.464 ± 0.917	-4.60% (0.0060)
PGMinvrpt(2)37A1	Male	59.855 ± 0.647	63.297 ± 0.656	+5.75% (0.0002)
PGMinvrpt(X)41A2	Male	83.443 ± 0.727	82.672 ± 0.948	-0.92% (0.52)
Control	Male	74.312 ± 0.941	70.947 ± 0.810	-4.53% (0.0066)

suggest that tet-on regulated overexpression and RNAi of genes near *Pgm* may be a promising approach.

Third, an interesting model is that Pgm^S contributes to increased lifespan of the selected strains because of an alteration in its regulation and/or subcellular localization. The sequence analysis of the *Pgm* alleles reveals a novel potential site in Pgm^S for phosphorylation by PKC; however, the potential significance of this change is unknown. The Pgm^S allele was used for the overexpression experiments presented here, and presumably the overexpressed enzyme should be subject to any regulation or localization characteristic of Pgm^S. However, the Pgm^S enzyme was overexpressed in a 'wild-type' Pgm^M background, and it may be that any phenotypic consequences of Pgm^S allele function are not apparent when Pgm^M is also present. Finally, another possible explanation for the absence of an effect on longevity is the method of assay. The selected O stocks were handled and assayed with males and females together, repeatedly mating during adult life. The present assay used segregated sexes, which is a different environment. This was done because the segregated-sexes assay is sensitive to even small changes in lifespan, and has been used successfully in the past to detect the effects of other genes on lifespan. It is possible that an effect of PGM on lifespan might be identified with different lifespan assay conditions. However, even if this explanation were to be true, the data still indicate that PGM does not have a simple strong effect on longevity. Further experiments will be required to distinguish between these interesting possibilities for the link between *Pgm* allele frequency and lifespan.

Materials and methods

Drosophila stocks and culture

All transgenic *Drosophila* stocks were generated by P-element germline transformation [43], using a modified microinjection technique [44]. Transgenic lines are named as the construct, followed by the chromosome of insertion in parentheses, followed by a unique line designation. For example, PGMinvrpt(3)76 is PGMinvrpt construct inserted on chromosome 3, independent transgenic line 76. Cytological sites of insertion were not determined. Multiple strains homozygous for each of the Pgm^F, Pgm^M and Pgm^S alleles were generated by purifying third chromosomes from the Ives stock, which is the precursor to the O and B selection stocks [19]. Homozygosity for a particular *Pgm* allele was confirmed by starch-gel electrophoresis assay, as previously described ([32] and data not shown). Other *Drosophila* strains are as described [45]. All stocks were grown on standard cornmeal-agar medium [46] and were cultured at 25°C.

Table 3

Mean lifespan at 29°C

Line name	Sex	Mean lifespan (29°C) -DOX	Mean lifespan (29°C) +DOX	Percentage change (*p*-value)
PGMsense(3)33A	Male	40.902 ± 0.834	43.910 ± 0.782	+7.35% (0.0092)
PGMsense(3)33B	Male	45.693 ± 0.581	44.663 ± 0.618	-2.25% (0.23)
PGMsense(2)43	Male	51.298 ± 0.626	53.989 ± 0.719	+5.25% (0.0050)
PGMsense(2)52	Male	49.874 ± 0.541	48.681 ± 0.647	-2.39% (0.16)
PGMsense(X)27A	Male	35.010 ± 0.470	36.030 ± 0.619	-2.91% (0.19)
PGMinvrpt(3)76	Male	48.827 ± 0.404	48.568 ± 0.621	-0.53% (0.73)
PGMinvrpt(3)23	Male	45.358 ± 0.606	47.726 ± 0.566	+5.25% (0.0046)
PGMinvrpt(2)12	Male	48.759 ± 0.647	51.646 ± 0.598	+5.83% (0.0011)
PGMinvrpt(2)37	Male	49.863 ± 0.583	51.826 ± 0.679	+3.93% (0.029)
PGMinvrpt(X)41	Male	41.747 ± 0.499	39.539 ± 0.981	-5.28% (0.046)

Lifespan assays

Strains homozygous for the transgenic constructs were crossed to rtTA(3)E2 strain, homozygous for the rtTA transactivator construct, to generate progeny heterozygous for both constructs [1]. Control flies were rtTA(3)E2/+, that is, lacking a target construct. To obtain adult flies of defined age, the crosses were cultured at 25°C in urine specimen bottles. Before eclosion of the majority of pupae, bottles were cleared of adults and newly eclosed flies were allowed to emerge over the next 48 h. Most of the males will have mated during this time. The males only were then removed and were designated one day old, and were maintained at 25°C at 40 per vial in culture vials with food, and passaged to new vials every 48 h. For certain experiments the adult males were maintained at 29°C, as indicated. For certain experiments, female flies were used, as indicated. Those flies being fed DOX were kept on food vials supplemented with 250 μg/ml DOX.

DNA sequencing

All DNA sequencing reactions were done using the dideoxy chain-termination protocol and T7 Sequenase v2.0 (Amersham). Multiple clones were sequenced for each of the Pgm^F, Pgm^M and Pgm^S alleles. The clones were obtained by polymerase chain reaction (PCR) amplification of each allele using genomic DNA template isolated from strains homozygous for each allele. The primers used in each amplification are as follows. The primer locations were assigned on the basis of their relative distance from the A in the ATG start codon, which was defined as position +1.

Pgm 5′-end-2 (-27 to -4): 5′-AGCCAGCAGCCGGAAAAC-TCCAGT-3′; *Pgm* 3′-end (+1706 to +1727): 5′-GGAT-GGGTTGGTAATCTCAGTG-3′. Each PCR product was gel-purified and cloned into the *Eco*RV site of pBluescript KS+ (Stratagene). Predicted protein motifs were identified using Prosite software [47].

Phosphogluconate mutase enzyme activity assays

The activity assay was carried out essentially as described [48]. Briefly, ten male flies were anesthetized with CO_2 and placed in 200 μl ice-cold 0.1 M Tris buffer solution (pH 7.6). The flies were homogenized using a motorized mortar and pestle. An additional 200 μl Tris buffer was added for a total volume of 400 μl. The sample was centrifuged for 20 min at 12,000*g* at 4°C. Approximately 200-300 μl supernatant was removed, taking care to avoid a top layer of oil and debris. For the assay, 30-40 μl of this supernatant was used in a 3.1 ml assay mixture that contained: 1.5 mM $MgCl_2$, 26 mM histidine solution (pH 7.6), 8 μM glucose-1,6-diphosphate, 0.45 U/ml glucose-6-phosphate dehydrogenase, and 0.2 mM NADP. This solution was mixed at room temperature and then 5 mM glucose 1-phosphate was added. The solution was mixed again and placed in a spectrophotometer. To insure that readings were being taken in the linear range of the enzyme activity, standard curves were generated with respect to both time and amount of protein added. Readings were taken at t = 6 min at OD_{340} and were expressed as ΔOD/μg/min. Protein concentrations were determined using the BioRad protein concentration solution using BSA as standard. All extracts were assayed in triplicate, and the averages and standard deviations are presented. Means were compared using unpaired, two-side t-tests and p-values are presented. The enzyme assay measures the conversion of $NADP^+$ to NADPH rather than a direct measure of phosphoglucomutase itself. In other words, it is measuring a secondary, coupled reaction.

DNA constructs

All inserts for microinjection were cloned into a derivative of the 7T40 construct [1] called USC1.0. USC1.0 was generated

as follows: First, 7T40 was digested with *Eco*RI, thus liberating the Hsp70 polyadenylation signal sequence. The construct was then religated and transformed. Next, the *Pst*I site in polylinker 1 was destroyed by partial *Pst*I digestion followed by T4 DNA polymerase fill-in and ligation. Clones were screened for destruction of the correct *Pst*I site to generate USC1.0 (Figure 2a). All inserts were cloned into the unique *Pst*I to *Eco*RI sites of USC1.0.

The *Pgm* sense construct was made as follows. The reverse transcription was carried out on total RNA isolated from a strain of flies homozygous for the Pgm^S allele, using the following primer: dTB_IB_O: 5′-TAACCCGGGTCTACAAAGTGATACTGCGTAACTGACTATATTTTTTTTTTTTTTTT-3′. A nested PCR strategy was then used to amplify the Pgm^S cDNA using the following primers: First primer set: B_I: 5′-TAACCCGGGTCTACAAAGTG-3′. *Pgm* 5′-end-2 (-27 to -4): 5′-AGCCAGCAGCCGGAAAACTCCAGT-3′. Second (nested) primer set: B_O: 5′-ATACTGCGTAACTGACTATA-3′. 5B6 (+1,520 to +1,534): 5′-CTGGAAGCTCGGGAG-3′. The resulting PCR product was cloned into the *Eco*RV site of pBluescript KS+ and clones were screened for the correct orientation. The Pgm^S cDNA was then liberated by *Eco*RI complete digest followed by *Pst*I partial digest and cloned into the *Eco*RI to *Pst*I sites of USC1.0 to generate construct PGMsense (Figure 2b).

The PGMinvrpt construct was made as follows: because the final insert would be a large inverted repeat, Pgm^S was PCR-amplified in two pieces, in opposite orientations. The two sets of primers used for the amplifications were: First set: *Pgm*-sense (3′) (+1,773 to +1,794): 5′-AGCTGAATTCCACAAACTTTAATAAATCCGAAAC-3′. *Pgm*-anti Not (+711 to +731): 5′-AGCTGCGGCCGCCCTGAACCGGTTGGGTGCCAC-3′. Second set: *Pgm*-anti Not (+711 to +731): 5′-AGCTGCGGCCGCCCTGAACCGGTTGGGTGCCAC-3′. *Pgm*-anti Pst (+1,773 to +1,794): 5′-AGCTCTGCAGCACAAACTTTAATAAATCCGAAAC-3′. Each of these PCR products was cloned individually into the *Eco*RV site of pBluescript KS+, and then restriction digested with *Eco*RI and *Not*I (first-set fragment) or *Not*I and *Pst*I (second-set fragment). Each of the liberated fragments was gel purified and triple-ligated into the *Pst*I to *Eco*RI sites of USC1.0 to generate construct PGMinvrpt (Figure 2c).

Northern analyses

RNA was extracted using Trizol reagent (Gibco BRL) and the amount of RNA was quantitated by UV spectrophotometry. Approximately 5-10 μg RNA per lane was resolved on a 1.0% agarose gel. RNA markers (Gibco BRL) were used to determine the sizes of bands. After blotting, the RNA was fixed to Genescreen nylon membrane (Dupont) using a UV crosslinker (Stratagene). Probes were ^{32}P-labeled using the Prime-It II kit (Stratagene). To determine fold increases/decreases, northern blots were exposed onto a phosphor screen and results were analyzed using a phosphorimager (ImageQuaNT, Molecular Dynamics). Ribosomal protein 49 gene (*Rp49*) [49] was used as a loading control, and all *Pgm* northern data is normalized to *Rp49* RNA levels.

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Functional Characterization of the Enhancer Blocking Element of the Sea Urchin Early Histone Gene Cluster Reveals Insulator Properties and Three Essential *cis*-acting Sequences

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Insulator elements can be functionally identified by their ability to shield promoters from regulators in a position-dependent manner or their ability to protect adjacent transgenes from position effects. We have previously reported the identification of a 265 bp *sns* DNA fragment at the 3′ end of the sea urchin H2A early histone gene that blocked expression of a reporter gene in transgenic embryos when placed between the enhancer and the promoter. Here we show that *sns* interferes with enhancer-promoter interaction in a directional manner. When *sns* is placed between the H2A modulator and the inducible *tet* operator, the modulator is barred from interaction with the basal promoter. However, the *tet* activator (tTA) can still activate the promoter, even in the presence of *sns*, demonstrating that *sns* does not interfere with activity of a downstream enhancer. In addition, the H2A modulator can still drive expression of a divergently oriented transcription unit, suggesting that *sns* does not inhibit binding of transcription factor(s) to the enhancer. To identify *cis*-acting sequence elements within *sns* which are responsible for insulator activity, we have performed *in vitro* DNase I footprinting and EMSA analysis, and *in vivo* functional assays by microinjection into sea urchin embryos. We have identified three binding sites for protein complexes: a palindrome, a direct repeat, and a C + T sequence that corresponds to seven GAGA motifs on the transcribed strand. Insulator function requires all three *cis*-acting elements. Based on these results, we conclude that *sns* displays properties similar to the best characterized insulators and suggest that directional blocking of enhancer-activated transcription by *sns* depends on the assembly of distinct DNA-protein complexes.

Keywords: histone genes; enhancer blocking; insulator; H2A enhancer; microinjection

Introduction

Proper temporal and spatial regulation of gene expression requires the orderly and efficient interaction of transcription factors with their cognate sites. The elucidation of how this might occur is one of the major challenges in molecular biology. If transcription units are organized into independent functional domains, enhancers could activate transcription from a promoter within the same domain but would be restrained from interacting with promoters in external domains. Insulators seem to be involved in the organization of the eukaryotic genome into domains of gene expression.[1–4] Insulators have been identified because they interfere with

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Abbreviation used: EMSA, electrophoretic mobility shift assay.

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enhancer-promoter communication only when positioned between them; for this reason they are generally called enhancer blocking elements. Although mechanisms by which these elements function in the normal context are not fully understood, it is thought that binding of chromosomal proteins to specific insulator sequences and subsequent protein-protein interactions can directionally restrict enhancer activity (for recent reviews see:[5,6]). The best characterized of these enhancer blocking elements are the *Drosphila*, scs, scs' and gypsy elements,[7–10] and the chicken HS4 insulator.[11,12] In addition to enhancer blocking activity, insulators can protect a transgene from chromosomal position effects when placed in flanking positions. However, only the chicken HS4[13,14] and the *Drosphila*, gypsy[15] elements seem to confer a barrier which prevents heterochromatin from spreading. Gypsy elements can also buffer a promoter from the silencing effect of the polycomb responsive element (PRE).[16] Elements which counteract silencing and act as heterochromatin boundaries have also been identified in yeast.[17–19] However, it is not known whether the yeast elements can also act as insulators of enhancer activity or display only chromatin boundary functions.[20]

Insulator DNA elements seem to be present in the tandemly repeated sea urchin early histone genes. The five early histone genes are expressed coordinately after meiotic maturation of the egg, and in early cleavage embryos until the blastula stage.[21] Although there is a single enhancer within the histone repeat unit, the 30 bp modulator of the H2A gene[22,23] each gene within the repeat is apparently regulated by gene-specific transcriptional elements..[24–26] From this observation, we hypothesized that there might be chromosomal elements which would direct and restrict H2A modulator function to its cognate H2A promoter. Subsequently, we identified a 265 bp sequence at the 3' end of the H2A early histone gene (see map in Figure 1), that, in microinjected sea urchin embryos, showed a blocking activity when placed between the enhancer and the promoter. We termed this sequence silencing nucleoprotein structure (*sns*). Although proof of the directionality of enhancer blocking activity was lacking, experimental evidence suggested that *sns* is an insulator of enhancer function rather than a general repressor of transcription. *sns* blocked enhancer-promoter interaction only when interposed, in either orientation, between a multiple array of the H2A modulator/enhancer and the basal thymidine kinase (tk) promoter. No other position influenced transgene expression. Furthermore, *sns* interfered with enhancer function but not with the activity of the basal promoter, in that it mantained the capacity to silence transgene expression when it was placed at a distance of 2.7 kb from the promoter.[28] Interestingly, our results suggested evolutionary conservation of enhancer blocking mechanisms, as *sns* was able to shield promoters from viral enhancers in transient and stably transfected human cell lines, as well as in *Xenopus laevis* oocytes[28] (unpublished observations).

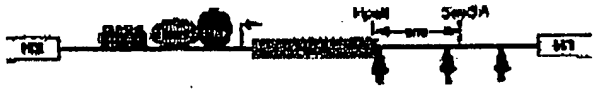

Figure 1. Map of the H2A transcription unit. CCAAT (CBF), modulator (MBF), putative GAGA factor binding sites in the H2A promoter, and the transcription start site are indicated. The *sns* fragment spanning from the *Hpa*II to the *Sau*3AI restriction sites starts 14 nucleotides upstream of the stop codon of the H2A gene and resides in a region where three micrococcal nuclease hypersensitive sites (vertical thick arrows) appear at gastrula stages.[27]

Here we have further characterized the properties of the sea urchin *sns* DNA fragment. We present evidence demonstrating that *sns*, as in the case of the best characterized insulators, affects only enhancers located distally from the promoter and displays a directionality in enhancer blocking activity. In addition, we report the identification of three protein binding sites within *sns* that, as demonstrated by functional assays in transgenic sea urchin embryos, are collectively required for insulator activity.

Results

sns interferes with enhancer function in a directional manner

Utilizing RNase protection assays, we previously demonstrated the ability of *sns* to inhibit enhancer-activated expression of a transgene in microinjected sea urchin embryos only when interposed between an array of the H2A modulator/enhancer and the *tk* promoter.[28] Because basal expression from *tk* or other viral promoters in sea urchin is negligible, effects on transcription were dramatic, with previously abundant transcript levels becoming undetectable. We have used the same approach to investigate whether *sns* evinces other behaviors expected of insulators. We placed *sns* between two enhancers in a chloramphenicol-acetyl transferase (CAT) gene transcription unit (Figure 2(a), construct 2). As a distal enhancer, we used the sea urchin H2A modulator array, containing at least four binding sites for the MBF-1 *trans*-activator; the proximal enhancer was the heptamerized tetracycline (*tet*) operator. In HeLa cells, the *tet* operator is induced upon binding of the Tet repressor-VP16 activation domain chimera (tTA *trans*-activator) and stimulates transcription of a reporter gene by several orders of magnitude.[29] The *tTA* gene was placed under the control of the multiple modulator elements and the *tk* minimal promoter (construct A). This construct was coinjected with the *CAT* reporter gene driven by the *tet*

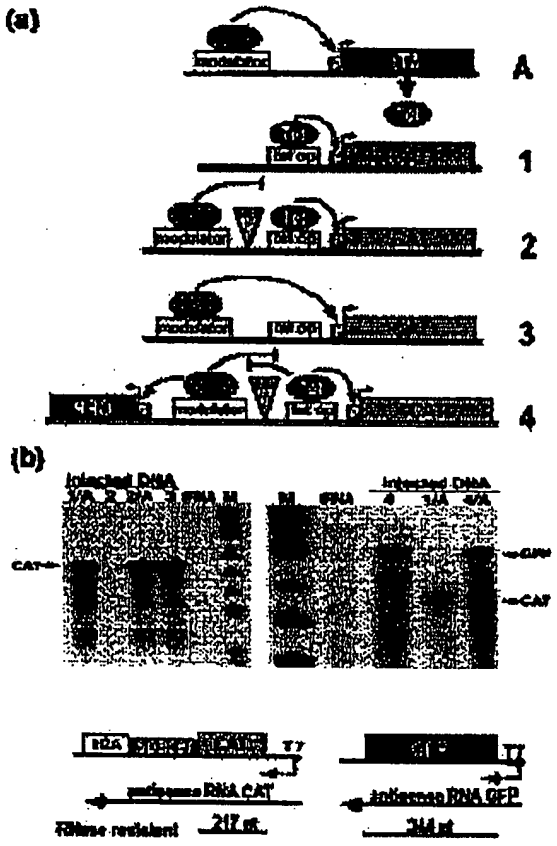

Figure 2. Polar and directional effects of *sns* on enhancer-promoter interaction. (a) Schematic representation of the microinjected plasmids. Binding of the MBF and the tTA transactivators, respectively, to the modulator and *tet* operator arrays is also indicated. Curved arrows refer to the activation of promoter by the bound factor; small arrows point to the transcription start site. (b) Total RNAs from microinjected embryos were processed to detect enhancer-activated transgene expression by RNase protection using antisense ^{32}P-labelled probes transcribed *in vitro* from the constructs illustrated below the gel images. Arrows point to the 217 nucleotide CAT and 344 nucleotide GFP RNase-resistant fragments. tRNA was used as negative RNA control. The RNase digestion products and end labelled *Hpa*II-digested pBluescript DNA (M) were run on denaturing polyacrylamide gels. Coinjection of constructs 1 and A (lanes 1/A) leads to CAT expression. In the absence of tTA, construct 2 is silent because the modulator is blocked by *sns* (lane 2). Coinjection of constructs 2 and A (lane 2/A) *trans*-activates *CAT* gene expression. Construct 3 expresses the *CAT* transgene because the *tet* operator does not interfere with the modulator (lane 3). Construct 4, in the absence of tTA, expresses only the GFP transgene (lane 4); if coinjected with A both the *CAT* and the *GFP* reporter genes are *trans*-activated (lane 4/A). Hence, *sns* does not block the binding of the transcription factors to the enhancers.

operator (construct 1). We predicted that expression of the tTA would elicit *trans*-activation of the transgene. As shown in Figure 2(b) (lane 1/A) this was indeed the case. Next, we tested the constructs with two enhancers. As expected, construct 2 containing *sns* between the MBF-1 and tTA binding sites was transcriptionally silent (lane 2), indicating that *sns* blocked the *trans*-activating function of the MBF-1 and that the *tet* operator was inactive in the absence of tTA. When the activator expression plasmid (construct A) was coinjected with construct 2, *trans*-activation of the transgene occurred (lane 2/A). The intensity of the CAT mRNA band detected in embryos injected with the two different plasmid combinations was almost identical (compare lane 1/A and 2/A), suggesting that neither the modulator nor *sns* sequences affected the extent of activation by tTA. In addition, the *tet* operator sequences did not interfere with the enhancer activity of the modulator, as similar levels of transgene expression were detected in embryos injected with construct 3 (lane 3). In summary, these experiments strongly suggest that *sns*, like chromatin insulators, has the ability to block the distal enhancer from communicating with the promoter but has no influence on the proximal one, when situated between the two.

We also investigated *sns* behavior in the context of a bidirectional transcription construct. A construct was made in which the modulator array and *tet* operator direct expression of two divergently transcribed reporter genes, encoding either CAT or green fluorescent protein (GFP). The *sns* sequence was inserted between the modulator and *tet* operator (construct 4 in Figure 2(a)). RNase protection assays were performed with RNA extracted from transgenic embryos, utilizing probes for both CAT and GFP in the same hybridization reaction. Only *CAT* mRNA was detected in embryos microinjected with constructs 1 and A (Figure 2(b), right panel, lane 1/A). As expected, in the absence of the tTA activator, the *CAT* transgene was not expressed in embryos injected with the bidirectional transcription unit (lane 4), presumably because *sns* interrupted the interactions between MBF-1 and the basal transcription apparatus. However, *sns* did not restrain MBF-1 from activating the divergent *GFP* transcription unit (lane 4). Subsequently, expression of tTA allowed for *trans*-activation of the *CAT* transcription unit (lane 4/A). From these results we conclude that *sns* blocks enhancer activity in a directional manner.

In vitro binding of proteins to *sns* sequences

To identify nuclear protein binding sites within *sns*, we performed DNase I footprint analysis and electrophoretic mobility shift assays (EMSA) with nuclear extracts from gastrula stage embryos. Two DNase I protected regions, defined as Box A and Box B, were mapped to both strands in the 5′ half of *sns* (Figure 3). The specificity of protein-DNA interaction was assessed by oligonucleotide compe-

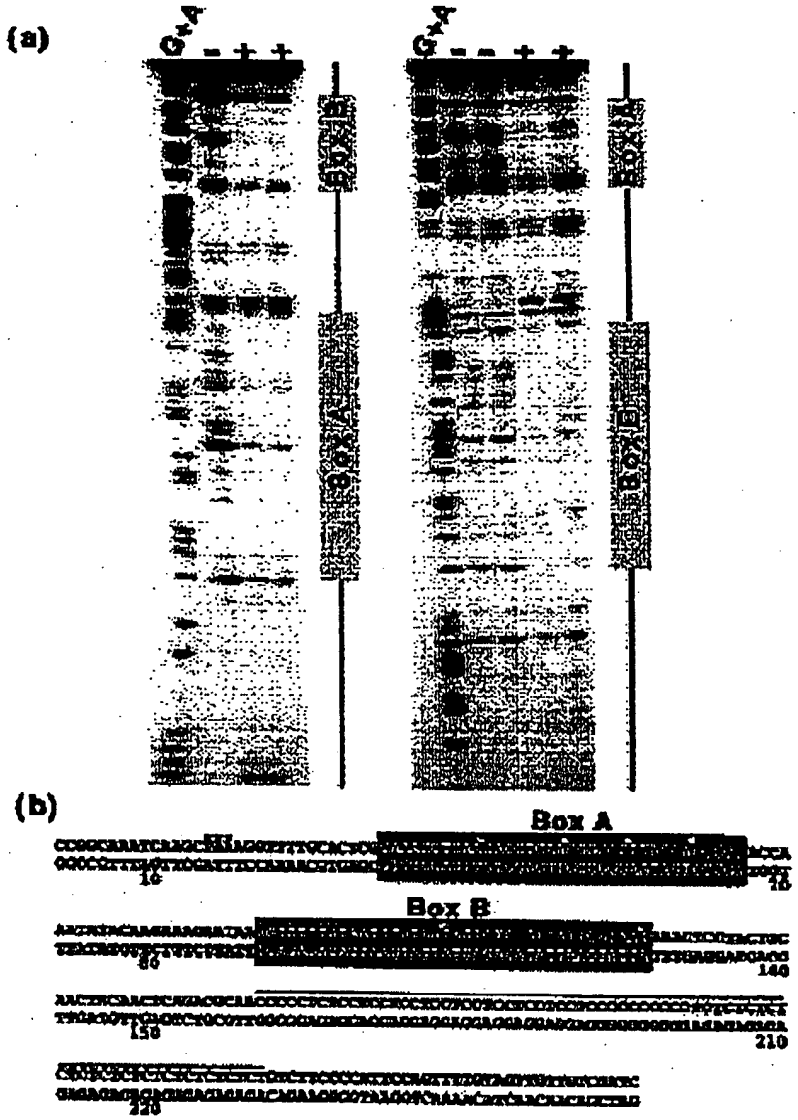

Figure 3. DNase I footprinting of an end-labelled *sns* sub-fragment spanning from nucleotides 2 to 157. (a) Sense strand (left) and antisense strand (right) were incubated with either BSA (lanes-) or nuclear extracts from gastrula stage embryos and digested with 5 μg of DNase I for three to five minutes on ice (lanes+). Digestion products were analyzed together with the cleavage products of the G + A sequence reaction on denaturing polyacrylamide gels. (b) Nucleotide sequence of the *sns* element. Asterisks mark the stop codon of the H2A gene. Sequences of protected Box A and Box B are in black boxes. Arrows inside boxes point to the Box A inverted and direct repeats and to the Box B direct repeat. Pyrimidine stretches are overlined.

tition experiments in EMSA. Figure 4 shows that both DNA-protein complexes were suppressed by an excess of unlabelled homologous probe, while they were not affected by an excess of unlabelled heterologous sequences. As indicated in the sequence shown in Figure 3(b) and in the drawing of Figure 4, Box A contains two notable sequence features: a C + A perfect direct repeat (DR), and immediately downstream the palindrome (IR) which is one of the *cis*-acting elements involved in 3′ RNA processing.[30] Because the IR sequence alone competed as efficiently as the entire Box A, we conclude that the palindrome is the protein binding site within Box A.

To search for further protein binding sites, we analyzed the pyrimidine stretch (C + T) at the 3′ end of *sns* sequences. This fragment contains 14 TC repeats that in the bottom strand correspond to seven GAGA sequences. EMSA analysis with nuclear extracts demonstrated that the C + T rich fragment formed a predominant DNA-protein complex that was specifically competed by an

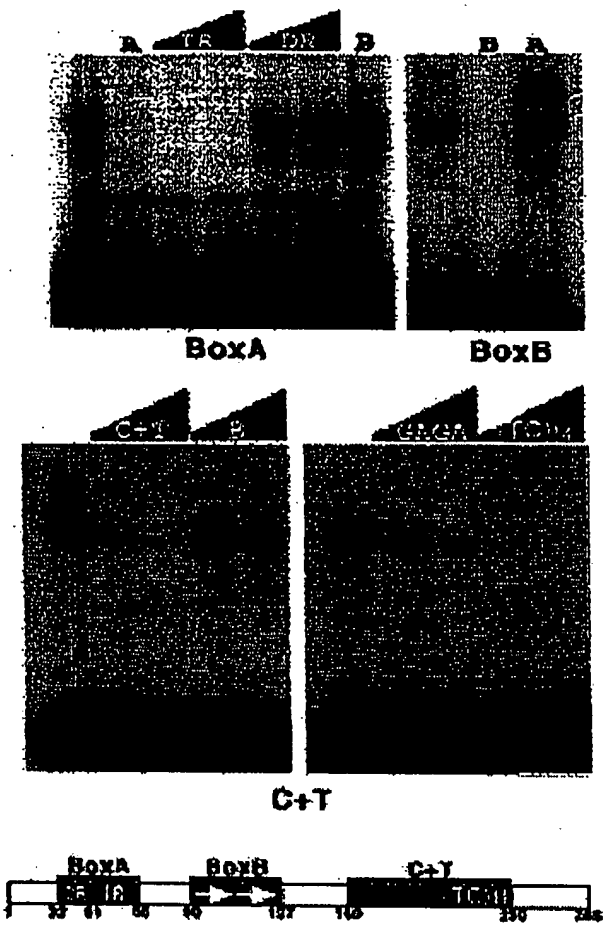

Figure 4. EMSA analysis of nuclear protein binding sites within *sns*. The three end labelled probes Box A, Box B and C+T, are underlined in the schematic drawing of the *sns* fragment. DR and IR refer, respectively, to the direct repeat and palindrome of Box A; white arrows in the Box B indicate a tandem repetition; (TC)14 refers to the 14 repetitions of the TC doublet. The C+T fragment was obtained by PCR amplification. All other probes were obtained by annealing complementary oligonucleotides. In competition experiments, nuclear extracts were pre-incubated with an excess of unlabelled homologous or heterologous probes prior to the addition of 1 ng of the labelled probe. The amounts used were: 100 ng for Box A (A) and Box B (B); 50 ng and 100 ng for IR, DR, C+T, GAGA (the GA repeats located upstream the H2A modulator), and (TC)14. The DNA-protein complexes were resolved by polyacrylamide gel electrophoresis.

excess of the homologous fragment (Figure 4). Of particular significance, protein binding was also specifically competed when nuclear extracts were pre-incubated either with an excess of a sequence containing four GAGA repeats, which is located upstream of the *H2A* modulator (see Figure 1), or with an oligonucleotide containing the 14 TC dinucleotides found at the 3′ end of the pyrimidine region (see sequence in Figure 3). The former competition was slightly less efficient, perhaps due to the presence of fewer (eight) TC dinucleotide repeats. These observations demonstrate the binding of nuclear protein(s) to GAGA sequences in sea urchin and suggest that a putative GAGA factor might contribute to the enhancer blocking function of *sns*.

Deletion of either the Box A palindrome or the 3′ CT repeats abolishes *sns* insulator function

We used the enhancer blocking assay to test the effect of 5′ and 3′ deletions of the *sns* fragment on the expression of a transgene driven by the *H2A* modulator in transgenic sea urchin embryos. The *sns* deletion mutants shown in Figure 5(a) were cloned between multiple copies of the 30 bp modulator/enhancer of the H2A histone gene and the tk promoter of the M30-CAT reporter plasmid (Figure 5(b)). Resulting constructs were microinjected into sea urchin eggs, embryos raised till gastrula stages and processed to determine *CAT* transgene expression by RNase protection analysis. Results depicted in Figure 5(c) are representative of several microinjection experiments. In agreement with our previous reports,[23,31] in the presence of one or several copies of the 30 bp histone H2A modulator sequence, transcriptional activation from the tk promoter occurs efficiently, as evidenced by abundant transgene transcripts (Figure 5(c), lanes 3, 9, 11). These M30-CAT constructs demonstrated once again the enhancer blocking function of the intact *sns* (lane 5). Deletions from either the 5′ or the 3′ that remove Box A(Δ sns), or the pyrimidine rich sequence (ΔIII sns), respectively, impaired the blocking activity of *sns* (lanes 4 and 13). In fact, levels of CAT transcripts were comparable to the construct lacking *sns* (lanes 3 and 11). As expected, 5′ deletions that

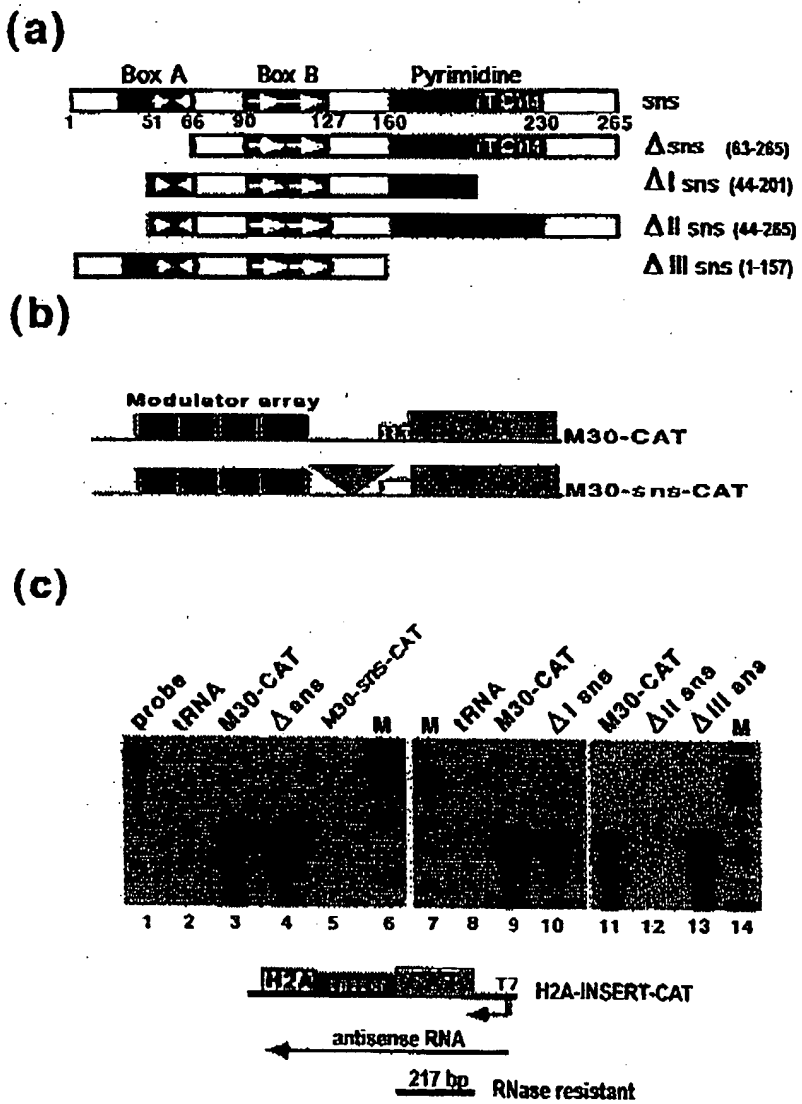

Figure 5. Functional activity of the *sns* deletion fragments. (a) Maps of *sns* and of the different deletion fragments assayed for enhancer blocking activity. (b) Schematic drawing of the microinjected plasmids. *sns* or the *sns* deletion fragments were inserted between the H2A modulator array and the tk promoter of M30-CAT to generate the M30-sns-CAT plasmids. (c) A ^{32}P-labelled antisense CAT RNA (lane 1), transcribed *in vitro* from the H2A-INSERT-CAT was used to perform RNase protection assays on microinjected embryos at gastrula stage. Electrophoretic analysis of the RNase digestion products was carried out on denaturing polyacrylamide gels. Lanes 3, 9 and 11: microinjection of the positive control M30-CAT to monitor enhancer-activated expression of the transgene. Lane 5: microinjection of M30-sns-CAT to monitor enhancer blocking activity of *sns*. Lanes 4, 10, 12 and 13: microinjection of reporter plasmids carrying, respectively, Δsns, ΔI sns, ΔII sns and ΔIII sns; only ΔII sns maintained the ability to attenuate the enhancer. Lanes 2 and 8: tRNA negative control. Lanes 6, 7 and 14: pBluescript *Hpa*II-digested end-labelled DNA markers.

left the palindrome intact, (ΔII sns) exhibited wild-type *sns* enhancer blocking activity (lane 12). Finally, removal of the TC repeats (ΔI sns) from ΔII *sns*, abolished the ability of *sns* to affect enhancer-promoter interaction (lane 10). Altogether, these results are consistent with the nuclear protein binding sites defined above and indicate that the Box A palindrome and the GAGA sites are essential for *sns* to block communication between the modulator and the tk promoter.

Box B is also essential for enhancer blocking activity

The experiments described in the previous sections suggest that the enhancer blocking function of *sns* relies on the assembly of protein complexes

at the Box A palindrome and at the GAGA sites. Because binding of proteins to Box B was also detected, we investigated whether these interactions were also essential for *sns* activity. Toward this end, we performed an *in vivo* competition experiment. We have previously used this approach to demonstrate that binding of the MBF-1 transcription factor to the modulator is required for activation of a transgene driven by the histone H2A promoter.[31] As indicated in Figure 6, sea urchin embryos were injected with the *sns*-containing transgene construct together with increasing amounts of ligated oligonucleotides containing either Box B (lanes 3, 4) or the Box A (lane 6) sequences. As levels of enhancer-activated transgene transcripts were similar to those seen with M30-CAT plasmid (lane 5), these results demonstrate that either oligonucleotide prevented enhancer blocking (lanes 2,7). Hence, titration of either Box A or Box B binding proteins by injecting their target sites impaired the ability of *sns* to block enhancer-promoter interaction.

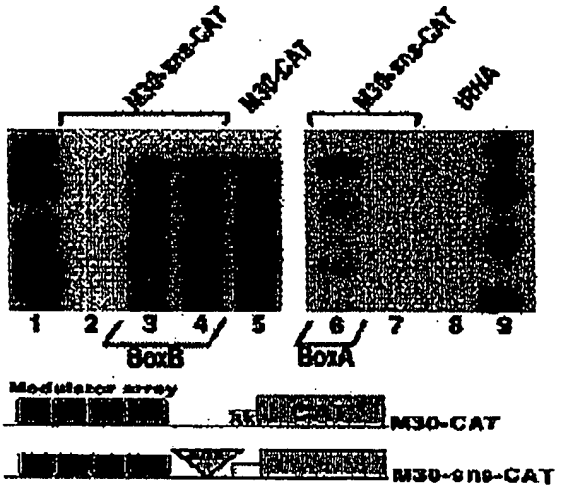

Figure 6. *In vivo* competition of *sns* function. Transgenic embryos were obtained by microinjecting the constructs drawn below the fluorograph with or without an excess of either BoxB or BoxA oligonucleotides. Black boxes represent the modulator array, the large shaded triangle the *sns* fragment. RNase protection experiments were carried out with total RNA as described in the legend to Figure 5. Lanes 2 and 7: injection of M30-sns-CAT; the enhancer is blocked. Lanes 3, 4 and 6: co-injection of M30-sns-CAT and 40-fold (lane 3) or 70-fold (lane 4) excess of ligated BoxB oligonucleotide or 70-fold (lane 6) excess of ligated BoxA oligonucleotide relieves the block. Lane 5: injection of M30-CAT; enhancer activity of the modulator array. Lanes 1 and 9 show relevant bands of DNA markers.

Discussion

Insulators are a new class of genetic elements that can modulate the activity of enhancer or other regulative sequences.[3,5] The few elements identified principally in *Drosophila* and chicken display two important characteristics: polarity and directionality of the effects of insulation of enhancer activity.[1,2] The former signifies that only enhancers located distally from the promoter with respect to the site of insertion of the insulator are attenuated in the interaction with the promoter. The second feature is that insulators do not prevent a blocked enhancer from activating transcription from a divergent promoter.[32,33] Consistently, we have shown that *sns* when placed between two enhancers, insulated the promoter-distal modulator without affecting the function of the downstream *tet* operator. In addition, *sns* did not interfere with the *trans*-activating capacity of the modulator in the other direction. Taken together, these results rule out that insertion of *sns* between enhancer and promoter represses enhancer-promoter interaction by enhancer inactivation, for example by inducing local assembly of a repressive chromatin structure.

As first shown in *Drosophila*, the directional enhancer blocking activity of insulator elements depends on the assembly of specific DNA-protein complexes. The gypsy insulator is perhaps the best-studied system with respect to the characterization of protein components that interact with insulator DNA. One of these components, the suppressor of Hairy-wing [su(Hw)] protein, binds to a reiterated target sequence[34] and recruits the second component, the mod(mdg4) protein[35] that displays properties characteristic of trithorax-group (trxG) genes.[36] The BEAF protein binds to the *scs'* insulator[37] which characterizes a class of chromosomal elements found at many loci.[38] Interestingly, seven tandem copies of an oligonucleotide containing BEAF binding sites has partial enhancer blocking activity.[37] The capability of the chicken HS4 insulator to interfere with enhancer-promoter interaction resides in a 42 bp fragment that contains a binding site for the CTCF transcription factor.[39] Binding of CTCF occurs also to several vertebrate insulators and to the unmethylated ICR (imprinting-control region) that displays enhancer blocking activity to control imprinted expression of the Igf2 gene.[40,41] Therefore, it is not surprising that the directional enhancer blocking activity of *sns* depends on specific DNA-protein interactions. It is of some interest that, while the enhancer blocking capacity of the *gypsy*, *HS4*, and to some extent the *scs'*, insulators relies on the recognition of a single or a reiterated binding site, *sns* contains three different *cis*-acting elements. Our results strongly suggest that all of these are needed to prevent enhancer-promoter interaction. In fact, deletion of either the Box A palindrome or the 14 TC repeats completely impaired *sns* function. Furthermore, microinjection of excess Box A or Box B and very recently GAGA (not shown) binding sites relieved the inhibition of the modulator in the *sns*-containing constructs. The most plausible explanation of the *in vivo* competition results is that the excess of

binding sites titrated, either directly or indirectly, the factors responsible for the enhancer blocking activity. Based on these observations, we speculate that *sns* achieves directional enhancer blocking activity by cooperative interactions between all three different DNA binding proteins or protein complexes.

Our results demonstrate that, within Box A, only the palindrome is required for enhancer blocking activity. Deletion of the 5′ most direct repeats, upstream of the palindrome, does not impair *sns* function. In agreement with this observation, oligonucleotides containing the direct repeats failed to compete for binding of factors to Box A and did not form specific protein-DNA complexes (not shown). Of some interest, the palindrome forms a stem-loop RNA structure, highly conserved among the non-polyadenylated histone mRNAs, from sea urchin, and *Drosophila* to mammals and represents one of the signals recognized by 3′ pre-histone mRNA processing machinery.[30]

A second *cis*-acting element was identified within the pyrimidine tract that contains seven GAGA repeats in the inverted orientation. Based upon EMSA analysis, specific protein interactions occur at the GAGA sites of *sns* and presumably at GAGA sites located upstream of the H2A modulator. Because the enhancer blocking activity of *sns* is independent of orientation,[28] it is reasonable to assume that protein(s) related to a *Drosophila* factor which binds GAGA sequences might be involved in the mechanism that interrupts the interaction between enhancer and promoter in sea urchin. *Drosophila* GAGA factor is a DNA binding protein involved in chromatin remodelling processes.[42] GAGA factor alleviates, in combination with NURF, the repressive effect of chromatin[43] and participates in the assembly of the silencing Polycomb proteins at PRE.[44] Interestingly, binding of factors to GAGA sites occurs in the spacer between the *Drosophila* H3 and H4 histone genes,[45] and recent evidence indicates a direct involvement of GAGA factor in insulator activity. GAGA factor binding sites, found at the PRE adjacent to the Fab-7 insulator, cooperate with Fab-7 to maintain the specific parasegment domain of expression of the Abdominal-B gene.[46,47] In addition, mutation of GAGA sequences within the insulator of the even-skipped locus affects directional blocking of the iab-5 enhancer.[48] Despite the similarity of the binding site and the apparent involvement in insulator function, the sea urchin protein differs from the *Drosophila* GAGA factor because a *Drosophila* polyclonal anti-GAGA factor antibody failed to supershift the *in vitro* assembled nuclear protein-DNA complex from sea urchin (not shown). The cloning of the sea urchin GAGA factor encoding gene should clarify whether the *Drosophila* and sea urchin factors are evolutionary and functionally related. One working hypothesis, currently under investigation, is that interactions between the proteins of *sns* and the proteins bound to the GAGA sites of the H2A promoter, prevent the H2A enhancer from acting promiscuously to activate transcription of heterologous early histone promoters.

With the exception of the GAGA element, the *sns* insulator sequence motifs are distinct from those described for other insulators. However, there is some evidence to suggest that these insulator sequences and their binding factors are also evolutionarily conserved. Very similar sequences are present in equivalent positions in the histone H2A transcription unit of the sea urchin *Psammechinus miliaris* (not shown). In addition, we have recently found that *sns* can insulate a viral enhancer upon stable integration in human chromatin (unpublished) and that at least two of the identified *cis*-acting insulator sequence elements, Box B and TC dinucleotide repeats (Box A did not show DNA binding activity in our conditions), interact specifically with human nuclear proteins of two different cell types (unpublished results).

In conclusion, we have extended our previous characterization of the *sns* element by the demonstration that *sns* acts equivalently to previously well-characterized insulators in a number of ways. We have now identified *cis*-acting sequences required for directional enhancer blocking activity, which may be evolutionarily conserved, and include novel sequences. Our studies have significant implications both for the control of early histone gene regulation in sea urchins, and for more general mechanisms of insulator action. In addition, these sequences may prove to have practical applicability in genetic engineering situations where insulator action might be beneficial.

Materials and Methods

Construction of plasmids

Plasmids, schematically drawn in Figure 2, were obtained as follows. Plasmid A that expresses the tTA activator, was constructed by the substitution of the CMV promoter of the pUHD 15.1 vector[29] with a fragment containing an array of the modulator sequences and the *tk* promoter. The pUHD 15.1 plasmid was digested with *Xho*I and *Xba*I simultaneously, filled in and ligated with a blunt ended DNA fragment containing the modulator sequences. Plasmid 1 that expresses the *CAT* gene under the control of the *tet* operator and the CMV promoter, was constructed by cloning the *tet* operator and the CMV promoter from the pUHD 10.3 plasmid[49] into the *Xho*I restriction site of the pBL-CAT3 vector.[50] Plasmid 3 that expresses the *CAT* gene under the control of two enhancers, the *tet* operator and the modulator, was obtained by cloning the 180 bp *Hin*dIII-*Xba*I DNA fragment containing the modulator repeats, derived from M30-CAT, in the *Hin*dIII-*Xba*I-digested plasmid 1. In construct 2, the *Hin*dIII-*Xba*I DNA fragment containing *sns* was cloned into plasmid 3 between *tet* operator and the modulator sequences. The EGFP gene (Clontech) fused to the *tk* promoter was cloned in inverted orientation upstream the modulator of plasmid 2, to generate the construct containing the two divergent transcription units (plasmid 4). To generate the plasmid M30-CAT an array of the H2A modulator/enhancer sequences was cloned into the *Sal*I site upstream of the

tk promoter of the pBL-CAT3 vector. To obtain the plasmid M30-sns-CAT, *sns* was inserted into the *Xba*I site of M30-CAT, between the modulator and the *tk* promoter. The *sns* fragment was isolated by *Hpa*II and *Sau*3A restriction enzyme digestion of the *Sac*I subclone of the PH70 histone DNA.[21] The Δsns, ΔI sns, ΔII sns and ΔIII sns deletion fragments were obtained from *sns* by restriction enzyme digestion or PCR amplification. These fragments were cloned respectively in the *Xba*I and *Bam*HI restriction sites located in the polylinker between the modulator and the tk promoter of the M30-CAT. The plasmids used for the RNase protection experiments, H2A-INSERT-CAT and GFP, were obtained as follows. The former contains a 258 bp pUC fragment inserted between the H2A promoter and a CAT coding subregion as described.[29] The latter bears a 344 bp fragment of the EGFP gene.

DNase I protection and electrophoretic mobility shift assays

Sense and antisense *sns* end-labelled fragments, spanning the region between nucleotides 2 and 157, were obtained by PCR. BSA or nuclear extracts from *P. lividus* embryos, prepared as described,[51] were preincubated with 10 μg of poly(dA-dT)·(dA-dT) in 50 μl of 10 mM Hepes (pH 7.9), 60 mM KCl, 1 mM DTT, 1 mM EDTA, 8% (v/v) glycerol for five minutes before the addition of the labelled fragments. After 20 minutes incubation, samples were digested with 5 μg of DNase I in 2.5 mM $MgCl_2$ for three to five minutes at 4°C, phenol extracted and loaded on 8% (w/v) denaturing polyacrylamide gels. In EMSA experiments, three different *sns* probes were assayed for binding activity *in vitro*: Box A, Box B double-stranded oligonucleotides (see next paragraph for sequences) and the C + T containing subfragment (spanning nucleotides 137 and 262) obtained by PCR. 1 ng of end labelled Box A , Box B and C + T probes were incubated, for 30 minutes on ice, with 5 μg of nuclear extracts from sea urchin embryos at gastrula stage and 2 μg of poly(dI-dC)·(dI-dC) in 20 μl of 10 mM Hepes (pH 7.9), 60 mM KCl, 1 mM DTT, 1 mM EDTA, 4% Ficoll. In the competition experiments, unlabelled homologous or heterologous probes in the amounts described in the legend to Figure 4 were added to the preincubation mixture prior to the addition of the extracts. Binding reactions were run on non-denaturing polyacrylamide gels in 50 mM Tris, 50 mM H_3BO_3 and 2 mM EDTA (pH 8.3).

List of oligonucleotides

Only the sense strand is reported. Residues of the following oligos refer to the first nucleotide of the *sns* sequence shown in Figure 3 (accession number Y09062): Box A: CAAACCTCAACACCTCAACGGCCCTTATCAGGGCCACCA (spanning nucleotides 32 to 70); Box B: AGTCTCTGTAATTCATAATAGTCTCTGTAATTCATAAT (spanning nucleotides 90 to 127); DR: CAAACCTCAACACCTCAAC (spanning nucleotides 32 to 51); IR: CTCAACGGCCCTTATCAGGCCCACCAA (spanning nucleotides 45 to 71); (TC)14:CCTCTCTCTCTCTCTCT CTCTCTCTCTCTCTGTCT (spanning nucleotides 200 to 234). The GAGA oligonucleotide, GGGAGGGAGAGAGAGAGAGAGA, spans nucleotides −200 to −177 from the transcription starting site of the H2A promoter.

Microinjection and transgene expression

Plasmids were linearized with *Hin*dIII or *Cla*I restriction enzymes whose sites are located in the polylinker regions, 5′ and 3′ to the CAT sequence, respectively. Linearized plasmids were brought to a total concentration of 240 μg/ml with threefold excess of *Hin*dIII-digested *P. lividus* sperm DNA of roughly 5 kb. Unfertilized eggs from a mature female were dejellied in pH 5.0 sea water, immobilized on Petri dishes with 1% (w/v) protamine sulfate and injected into the cytoplasm with 1-2 pl DNA in 25% glycerol solution. Microinjected eggs were fertilized and embryos raised up to the early gastrula stage (14 hours development). 3000 uninjected embryos at the same developmental stage were then added as carrier, and embryos collected by low speed centrifugation. Total RNA was extracted by one hour incubation at 55°C in a solution containing 200 μg/ml Proteinase K and phenol extraction. Nucleic acid samples were digested with RNase-free DNase I, and RNA from 50 microinjected embryos was hybridized with [^{32}P]UTP-labelled antisense CAT or EGFP RNA transcribed *in vitro* from the H2A-spacer-CAT and GFP plasmids. Hybridisation conditions, RNase digestion and gel fractionation of the RNase-resistant hybrids were as previously described.[52]

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Tetracycline-Regulated Gene Expression Switch in *Xenopus laevis*

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***Xenopus* is a well-characterized model system for the investigation of biological processes at the molecular, cellular, and developmental level. The successful application of a rapid and reliable method for transgenic approaches in *Xenopus* has led to renewed interest in this system. We have explored the applicability of tetracycline-regulated gene expression, first described by Gossen and Bujard in 1992, to the *Xenopus* system. By optimizing conditions, tetracycline repressor induced expression of a luciferase reporter gene was readily and reproducibly achieved in both the *Xenopus* oocyte and developing embryo. This high level of expression was effectively abrogated by addition of low levels of tetracycline. The significance of this newly defined system for studies of chromatin dynamics and developmental processes is discussed.** © 2000 Academic Press

Key Words: **Xenopus; chromatin; transcription; development; gene expression; tetracycline.**

INTRODUCTION

The *Xenopus* oocyte and embryo have provided powerful model systems for the elucidation of mechanisms governing cellular and developmental processes [1–4]. In the case of the embryo, the description and use of a rapid and reliable *Xenopus* transgenic approach [5–8] have given a new perspective to *Xenopus* developmental studies [9]. This approach permits the overexpression of gene products in every cell of the organism or in a specific tissue. With this new technology comes the need to define parameters for its effective application, including the adaptation of existing transgenic methodologies. One of the invaluable features of the *Xenopus* oocyte system is its capacity to efficiently transcribe foreign genes encoded on microinjected plasmids following their assembly into chromatin [1, 10–13]. Recent evidence has suggested that processes regulating chromatin stability are linked to the transcriptional regulatory machinery (reviewed in [14]), highlighting the need to examine nuclear processes in a chromatin context. Since both transcription and replication can be assessed on templates assembled into chromatin using the *Xenopus* system, regulatory tools for use in the *Xenopus* oocyte or embryo would have important applications for the study of interactions between chromatin and the transcription and replication machinery. One such regulatory tool is the tetracycline-mediated gene expression switch.

Tetracycline-controlled gene expression was first described by Gossen and Bujard in 1992 and utilizes the very specific and high affinity binding of the *E. coli* tetracycline repressor protein (tetR) to its operator sequence (tetO) [15]. Using a fusion protein consisting of tetR fused to the VP16 activation domain (tTA) in HeLa cells, a luciferase reporter gene was activated up to five orders of magnitude and "turned off" to basal levels by the addition of low amounts of tetracycline to the tissue culture media. This tightly regulated genetic switch has been employed in a variety of studies where conditional gene expression is required. It has been used successfully in transgenic mice [16–18], where it is particularly appealing when the gene products under study are toxic or inhibitory to embryonic development. To address more diverse questions, the effective binding of tetR to its operon sequence has been exploited for purposes other than gene regulation. For example, it has been used in yeast to mark a specific region of DNA for mapping of sister chromatid separation with a tetR/GFP fusion protein bound to an array of tetO sites [19–21]. In the context of chromatin, tetR was demonstrated to form a physical boundary to nucleosome mobility in an *in vitro Drosophila* assembly system, thereby establishing a means to functionally analyze the chromatin remodeling machine CHRAC [22]. Here we describe, for the first time, the optimal conditions for successful application of this tetracycline-regulated switch for *in vivo* approaches in both the *Xenopus* oocyte and embryo.

MATERIALS AND METHODS

Antibodies and Constructs

pUHC13.3 contains seven tetO binding sites upstream of the minimal CMV promoter driving the luciferase reporter gene and pUHD15.1 expresses tTA protein. Both were generous gifts from S. Robine and have been previously described [15]. tTA mRNA was transcribed *in vitro* from the pSP65tTA plasmid constructed by in-

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serting the tTA coding region from pUHD15.1 into pSP65 by *Eco*R1/*Bam*H1 digestion and ligation. M13E4tetO was produced by removing the five Gal4 binding sites from M13E4G5 [2] by a *Hin*dIII/*Bam*HI digest and blunt ligation to the seven tandem repeats of tetO removed from pUHC13.3 by *Xho*I/*Stu*I digestion. The sequences and orientation of constructs were confirmed by sequencing. The TetR monoclonal antibody was raised against the tetracycline-responsive transcriptional activator tTA (Clontech catalog no. 8632-1) and was used at a 1:500 dilution for Western blotting according to standard protocols.

Xenopus Microinjection Strategy

Stage VI *Xenopus* oocytes were surgically removed and treated with collagenase as previously described ([11]; for methodological reviews, see [23]). *In vitro* transcribed tTA mRNA (quantity and quality assessed by UV analysis and electrophoresis) was injected into the cytoplasm of stage VI oocytes using a Drummond Nanoject automatic injector and incubated at 16°C overnight to allow tTA protein expression and accumulation. Approximately 18 h later, the tTA-regulated luciferase reporter pUHC13.3 [15] was injected into the nucleus and oocytes were incubated +/− 200 ng/ml tetracycline hydrochloride (Sigma) for a further 5 h to allow time for chromatin assembly on the reporter plasmid and luciferase expression. Ten healthy oocytes were recovered and lysed in 100 μl of lysis buffer, and the levels of luciferase reporter activity were assessed as described in the luciferase detection kit (Perkin–Elmer). Following lysis, DNA and RNA were analyzed as previously described [2].

Fertilized *Xenopus* eggs were coinjected into one blastomere at the 2-cell stage of development with 50 pg of pUHC13.3 reporter DNA and various amounts of tTA mRNA in a total volume of 26.7–32.2 nl as previously described [3]. Embryos were incubated +/− 2 μg/ml tetracycline hydrochloride for at least 14 h at 23°C. Alternatively, tetracycline was dissolved in water and coinjected into the embryos with pUHC13.3 and tTA mRNA to give a final concentration in the embryo of approximately 500 ng/ml. Embryos were lysed at various stages of development [24] and luciferase activity assessed.

Transcription Analysis

To assess the levels of either luciferase or E4 transcript accumulated per DNA template, a reverse transcription assay was performed as previously described [2]. Accumulation of luciferase mRNA transcript from 5 ng/oocyte pUHC13.3 luciferase reporter was detected by reverse transcription from an end-labeled oligo(5′-AGCCTTATGCAGTTGCTCTC-3′) annealed to luciferase mRNA extracted from the oocyte as previously described [2]. Extension gives rise to a product of 306 nucleotides. The same method was used to detect the E4 transcript, using in this case the end-labeled oligo(5′-CTTCACACCGGCAGCCTAACAGTCAGCC-3′), which produces a major product of 100 nucleotides. Efficiency of microinjection and chromatin assembly on transcribed templates was assessed by performing a supercoiling assay in parallel on DNA extracted from the same lysate used to prepare the RNA for reverse transcription analysis. Each lane of gel represents extension product from RNA accumulated in 2 oocytes. Quantification of transcripts and DNA recovery was performed with a PhosphoImager (STORM).

Supercoiling Assay

Following lysis of oocytes, a supercoiling assay was used as a measure of the efficiency of microinjection and assembly of injected templates into chromatin as previously described [2]. Each lane of the supercoiling assay represents DNA extracted from 5 oocytes from an injection of 5 ng/oocyte. Following electrophoresis, the DNA was Southern blotted and hybridized to M13E4tetO radioactively labeled with ^{32}P by random priming (Amersham Rediprime II RPN 1633).

RESULTS

Tetracycline-Regulated Gene Expression in the Xenopus Oocyte

It has been reported that the responsiveness of tetracycline-inducible systems can vary, depending on the cell type [25, 26]. In order to determine whether conditional gene expression could be achieved in *Xenopus* using the tetracycline-regulated system, we first asked whether expression of a luciferase reporter could be induced by tTA using a transient assay. The tTA protein was produced in the oocyte following cytoplasmic microinjection of *in vitro* transcribed tTA mRNA. This approach has previously been reported to be a reliable method for introducing foreign proteins into the oocyte [27–29], where the activity of injected RNA may be further improved by flanking the RNA with untranslated regions of the *Xenopus* globin gene. Therefore, we determined the activation threshold using 5 ng of microinjected reporter DNA when coinjecting increasing amounts of tTA mRNA. First, we confirmed that tTA protein was being produced in the oocyte at levels corresponding to the increase in injected mRNA (Fig. 1A). Second, we measured accumulation of luciferase mRNA by a reverse transcription assay (Fig. 1B). When the level of luciferase transcript was assessed, it was evident that an increase in activation of transcription from this reporter could be obtained using up to 75 ng of tTA mRNA, with 5 ng of reporter DNA reaching an optimal induction between 60 and 75 ng (Fig. 1B). The amount of tTA mRNA required to reach this optimal level is within the previously reported limit of 100 ng of mRNA that can be expressed by a single oocyte [30]. Factors available for luciferase protein expression also appear to be limiting since luciferase activity reaches a peak at 45 ng of injected tTA RNA (Fig. 1C), indicating that when high quantities of DNA are required, transcription efficiency should be assessed by analysis of the resulting transcript rather than the protein product.

Luciferase activity from the pUHC13.3 reporter was subsequently used as a measure of tTA-regulated expression of low levels of reporter since it has been well characterized for transient assays in tissue culture cells [15, 31, 32]. Figure 2A illustrates the experimental strategy taken for introduction of the tTA protein and luciferase reporter and analysis of products in the *Xenopus* oocyte. With this strategy, a range of amounts of both reporter plasmid and tTA mRNA gave high levels of reporter induction (Fig. 2B), achieving almost 50-fold induction of luciferase activity above basal levels expressed from the reporter without the tTA activator. An optimal induction was reached when 250 pg of reporter and 15–22 ng of tTA mRNA were microinjected since no significant increase in activation was

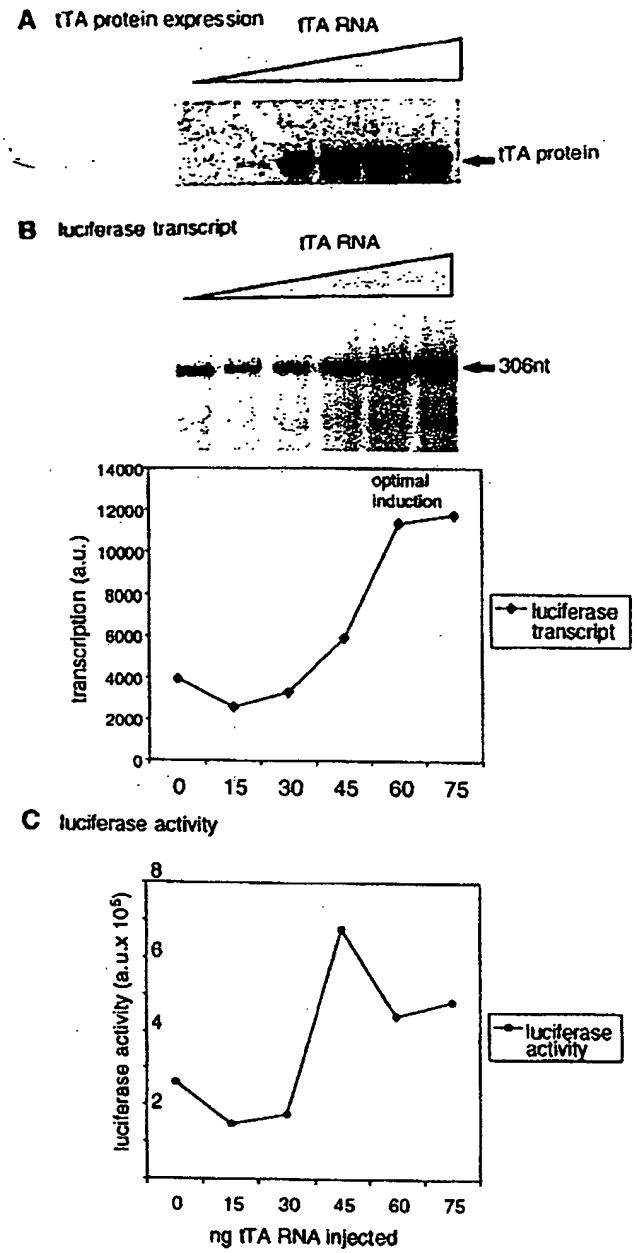

FIG. 1. Optimizing conditions for microinjection of tTA mRNA in the *Xenopus* oocyte. (A) Western analysis of *in vivo* translated tTA protein from oocyte extracts. Stage VI *Xenopus* oocytes were injected into the cytoplasm with the following increasing amounts of *in vitro* transcribed tTA mRNA: 0, 15, 30, 45, 60, and 75 ng/oocyte. Following injection, oocytes were incubated overnight for tTA protein expression and accumulation. Fifty oocytes were homogenized for each variable and centrifuged at 35,000 rpm and the clear protein extract layer was removed. Extract from the equivalent of 2 oocytes was then analyzed by Western blotting and revealed using a TetR monoclonal antibody (Clontech catalog no. 8632-1) at a 1:500 dilution and chemiluminescence according to manufacturer's instructions (Pierce SuperSignal, catalog no. 34080). (B) tTA-activated transcription from the pUHC13.3 reporter. (upper panel) Transcription of luciferase mRNA from 5 ng of pUHC13.3 luciferase reporter was detected by reverse transcription as described under Materials and Methods. The expected size of the reverse transcription product is given at 306 nt. A range of tTA mRNA of 0, 15, 30, 45, 60, and 75 ng/oocyte was injected in each case. (lower panel) Graphic illustration of accumulated luciferase mRNA, as described above, analyzed by phosphoimaging of the upper panel reverse transcription reaction. Optimal induction is indicated. (C) tTA-activated luciferase activity. Expression of luciferase from 5 ng of pUHC13.3 luciferase reporter was measured by a luciferase assay as described under Materials and Methods. A range of amounts of tTA mRNA of 0, 15, 30, 45, 60, and 75 ng/oocyte was injected as indicated.

detected despite the increased tTA RNA injected. This suggests that at this level all tTA binding sites are occupied. Significantly, addition of 200 ng/ml of tetracycline to the culture media was sufficient to completely abrogate this induction (Fig. 2B). Therefore, low levels of injected reporter DNA produced conditional gene expression, illustrating the effectiveness of this regulatory system in the *Xenopus* oocyte.

Tetracycline-Regulated Gene Expression during Development

Because of the high specificity and the low toxicity of the tetR protein and the tetracycline effector [15, 16], we wanted to determine the effectiveness of this system for use in the developing *Xenopus* embryo. Using an experimental strategy depicted in Fig. 3A, we coinjected fertilized eggs with luciferase reporter and various amounts of tTA mRNA to define the optimal parameters for tTA-driven promoter activation. Levels of injected DNA were kept to a minimum (50 pg/embryo) since higher amounts of DNA injected during early development result in low survival rates [33]. The most effective level of tTA mRNA to coinject with 50 pg of reporter was assessed at two different stages of development and determined to be 15–22 ng/embryo (data not shown). With these optimal conditions, high and reproducible levels of conditional gene expression were achieved in the developing embryo (Fig. 3B) following the midblastula transition when zygotic transcription is initiated (reviewed in [34]). At stage 11, although there is background luciferase activity expressed from the reporter plasmid, indicating that the embryos have developed beyond the MBT, there is no evidence of induction of luciferase expression by tTA, suggesting that not enough time has elapsed to establish levels of tTA protein necessary for activation of transcription. By stage 12, however, there is an effective induction of luciferase expression by tTA to over 100-fold, which is maintained through early development, with the peak of activation at stage 19 producing over 200-fold stimulation above levels with the reporter alone. By stage 27, the level of activation has dropped to 74-fold, indicating that the maximal limits of the system may have been reached by this stage. Significantly, levels of injected DNA are reported to decline following gastrulation [35], which may reflect the inability of even high levels of injected

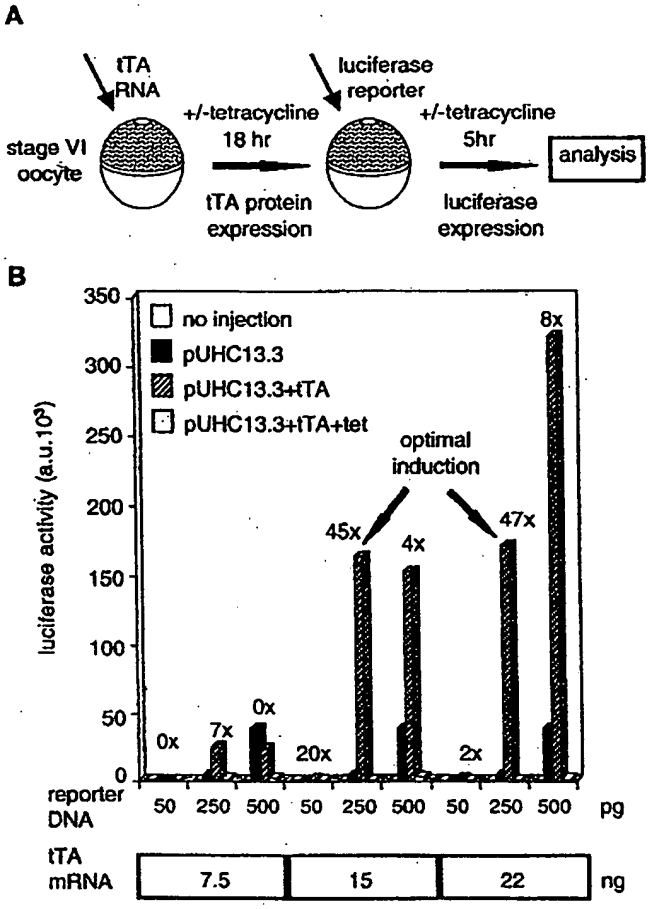

FIG. 2. Tetracycline-regulated gene expression in the *Xenopus* oocyte. (A) Experimental strategy for the tetracycline-regulated gene expression system in the *Xenopus* oocyte. Stage VI *Xenopus* oocytes were surgically removed and treated with collagenase as previously described [11, 23]. *In vitro* transcribed tTA mRNA was injected into the cytoplasm of oocytes, which were subsequently incubated at 16°C overnight +/− 200 ng/ml tetracycline hydrochloride (Sigma) to allow tTA protein expression and accumulation. Approximately 18 h later, the tTA-regulated luciferase reporter pUHC13.3 [15] was injected into the nucleus of the same oocytes and incubation continued +/− tetracycline for a further 5 h to allow time for chromatin assembly on the reporter plasmid and luciferase expression. (B) tTA induction of luciferase activity in *Xenopus* oocytes. Histograms illustrate the levels of luciferase activity, in relative light units, detected in the equivalent of 2 oocytes from the lysis of a total of 10 healthy oocytes per variable. Levels of induction and its abrogation on addition of 200 ng/ml tetracycline are shown after injecting 50, 250, or 500 pg of pUHC13.3 luciferase reporter and either 7.5, 15, or 22 ng of tTA mRNA/oocyte as indicated. Optimal induction and fold activation +tTA are indicated.

tTA mRNA to increase luciferase expression at this time. The above results indicate that the optimal induction of gene expression driven by the tTA protein using this transient assay is obtained at stage 19 of development, with high conditional activation achieved from stages 12 through to at least 27 using a ratio of 1:440 coinjected reporter DNA to tTA mRNA. We confirmed that the injected mRNA was translated into tTA protein by Western blotting of embryo extracts and that this level of mRNA was not inhibitory to early embryonic development (Fig. 3C). Injected DNA has been shown to persist for many months at low levels in the developing *Xenopus* [33, 35] probably following its integration into the host cell genome, suggesting that a mosaic pattern of induction may be achievable even at advanced stages of development using the protocol outlined in Fig. 3A. In fact, we were able to detect some activity from the luciferase reporter to the swimming larvae stage (stage 43) (data not shown).

Although it is clear that tTA can reach its tetO binding site to activate transcription in the embryo, results from these experiments indicate that tetracycline in the culture media has no effect on this activation (Fig. 3B, gray histograms). In a subsequent tetracycline titration experiment, a similar lack of effect was observed using 200 ng/ml and 0.02 mg/ml tetracycline (data not shown). Very high levels of tetracycline caused an arrest in development (at stage 13 for 0.2 mg/ml and stage 7 in the case of 2 mg/ml). Although the tetracycline-mediated shutoff of gene expression by tTA can be effectively achieved in tissue culture cells [15, 30] and in the *Xenopus* oocyte (Fig. 2B), it is not as rapidly established in whole organisms. Tetracycline can reduce levels of tTA-induced expression in transgenic mice implanted with slow-release tetracycline pellets, but this is often measured following 7 days of implantation [16] and variations in effectiveness with tissue type have been shown to exist [16–18]. We reasoned that *Xenopus* embryos could be more resistant than oocytes to the diffusion of tetracycline from the culture media. To address this question, we coinjected tetracycline with tTA mRNA and reporter DNA rather than adding tetracycline to the culture media. With this protocol, tTA-induced activation of luciferase was abolished by tetracycline even though injection of tetracycline with the reporter alone did not change the basal levels of luciferase expression (Fig. 4). The elimination of luciferase activity by tetracycline injection (compare pUHC13.3 + tTA histogram to pUHC13.3 + tTA + tet histogram) is remarkable considering the lack of tetracycline-mediated shutoff detected in Fig. 3B at a similar stage of development (stage 12, gray histogram). The tetracycline-regulated release of tetR from its recognition site can thereby be achieved using this coinjection strategy; however, optimization of this parameter will be needed to fully exploit the tetracycline-regulated system in the embryo.

Chromatin Assembly Coupled to Second-Strand Synthesis Eliminates Background Expression

To regulate gene expression in either the oocyte or as an integrated gene in a transgenic frog, the tTA protein must be competent to access its binding site on a DNA

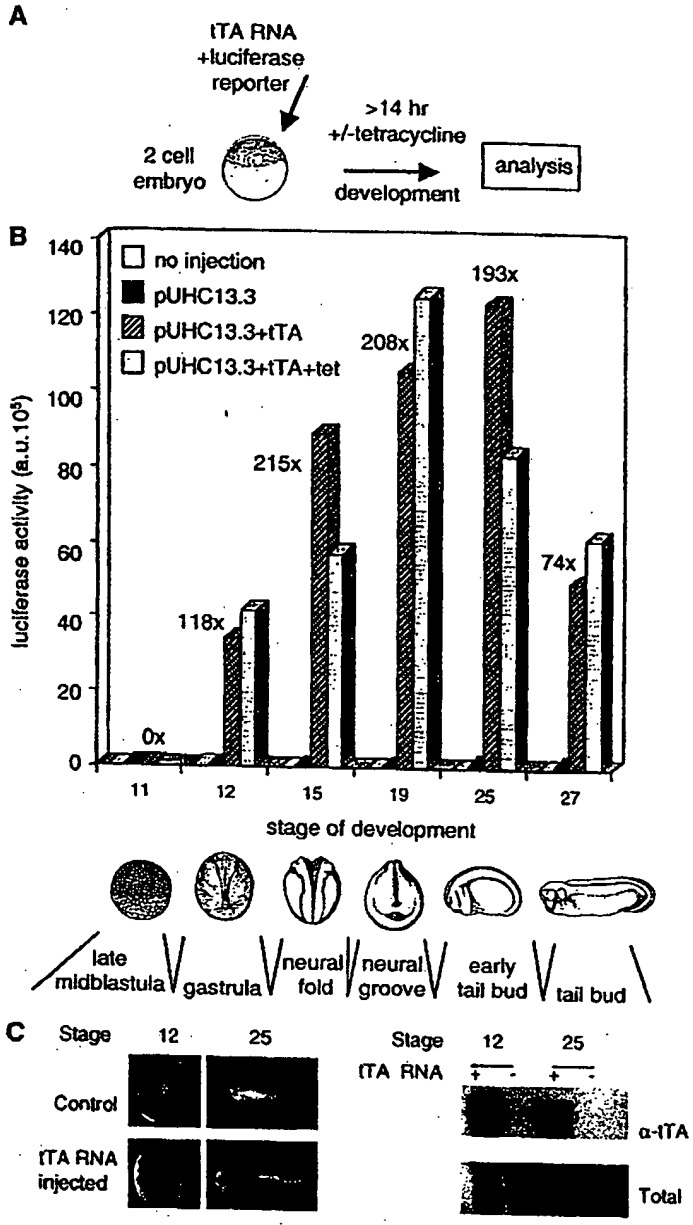

FIG. 3. Tetracycline-regulated gene expression during *Xenopus* development. (A) Experimental strategy in developing embryos. Fertilized *Xenopus* eggs were coinjected into one blastomere at the 2-cell stage of development with 50 pg of pUHC13.3 reporter DNA and various amounts of tTA mRNA in a total volume of 26.7–32.2 nl as previously described [3]. Embryos were incubated +/− tetracycline hydrochloride for at least 14 h at 23°C and lysed at various stages of development [24] to assess luciferase activity. (B) tTA induction of luciferase expression in the developing embryo. Histograms illustrate luciferase activity, in relative light units, detected from 2 embryos at various stages of development [24] as defined beneath (diagrams from Xenopus Molecular Marker Resource at http://vize222.zo.utexas.edu/). In each case, 22 ng of tTA RNA and 50 pg of pUHC13.3/embryo were coinjected and embryos incubated +/− 2 μg/ml tetracycline. Fold of activation +tTA is indicated for each developmental stage. (C) Expression of the tTA in the developing embryo. Left: pictures of control (top) and injected embryos with 25 ng of tTA RNA (bottom) were taken at the stages 12 and 25 according to [24]. Right: Western blot analysis of the corresponding embryos.

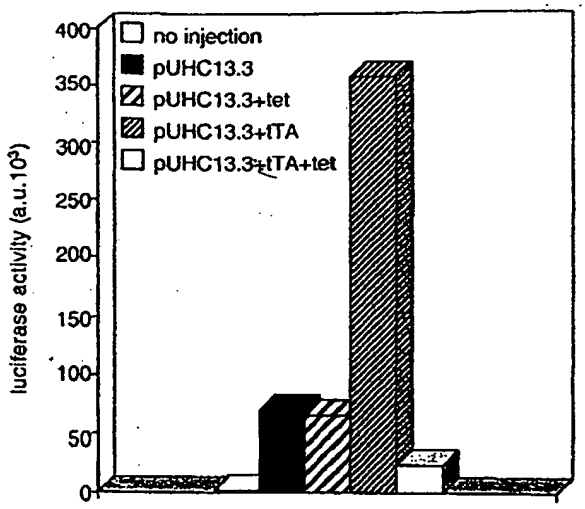

FIG. 4. Microinjection of tetracycline to regulate tTA binding. Histograms illustrate luciferase activity, in relative light units, detected from 3 embryos at stage 11–12 (midblastula) of development. In each case, 50 pg of pUHC13.3 and/or 22 ng of tTA mRNA per embryo was coinjected. Some samples, as indicated, were coinjected with a tetracycline solution to give a final concentration in the embryo of approximately 500 ng/ml.

template assembled into chromatin. Therefore, to extend the use of the induction system for analysis of microinjected reporter DNA assembled into chromatin, we further refined the conditions. Higher amounts of reporter DNA are required in this case since 1–5 ng of injected double-stranded plasmid DNA is the minimum amount necessary for effective chromatin assembly in the *Xenopus* oocyte [36]. We know from our initial experiments (Fig. 1) that 5 ng of reporter DNA is optimally activated by injection of 60–75 ng of tTA mRNA, indicating that tTA-regulated gene expression occurs on chromatinized templates, illustrating its potential as a tool either for regulation of integrated genes in transgenics or for *in vivo* chromatin studies. However, when we increase the amount of reporter DNA, background expression of luciferase is detected concomitant with a decrease in the activation potential (see Fig. 2B, 500-pg level, and Fig. 1B, basal transcription level). The high level of basal transcription from the minimal CMV promoter in this reporter construct is not surprising since genes driven by the CMV promoter are known to be highly expressed in the oocyte [2]. To eliminate this background expression from the reporter and to assess the effectiveness of this system in an integrated gene, we initiated a single-stranded (ss) template strategy. It has been shown that ss tem-

The tTA protein is detected using the TetR monoclonal antibody (top panel) and the total protein is detected using Ponceau staining (bottom panel). The equivalent of 2 embryos is loaded in each lane.

plates injected into oocytes are assembled into chromatin during the process of second-strand synthesis, which is repressive to basal transcription (initially reported in [2] and subsequently applied in [12, 29, 37, 38]). We reasoned that the introduction of the tTA-driven promoter on a ss template would provide a much more tightly regulated on/off system by repressing the effects of basal transcription. In addition, the ss template, once assembled into chromatin in the oocyte, would produce a template with the characteristics of an integrated promoter. Therefore, we constructed an M13 derivative (M13E4tetO) by removing the seven tetO binding sites from pUHC13.3 and inserting them into M13E4G5 [2] in place of the five Gal4 binding sites. Using this construct, we can compare both basal and tTA-activated transcription of the E4 gene during second-strand synthesis coupled chromatin assembly using a reverse transcription assay (Fig. 5A). When the ss template is injected, basal transcription is not detected (lane 1) whereas the double-stranded (ds) template shows background expression (lane 2) as expected. Although both the ss and ds templates were assembled into chromatin (as indicated by supercoiling, Fig. 5B), the tTA protein was able to overcome this chromatin-repressed state and activate transcription (lanes 3 and 7 for ss template and lanes 4 and 8 for ds template). Addition of 200 ng/ml of tetracycline to the oocyte culture media turned off the expression of the E4 gene (lanes 5 and 9 for ss template and lanes 6 and 10 for ds template). Therefore this ss template strategy provides a tightly regulated system to examine questions related to transcription from chromatin templates in the oocyte. It also indicates that the tetO binding sites would be accessible to tTA protein in an integrated promoter and that basal expression would be eliminated, providing an attractive mechanism for regulating overexpression of otherwise deleterious gene products in a transgenic *Xenopus*.

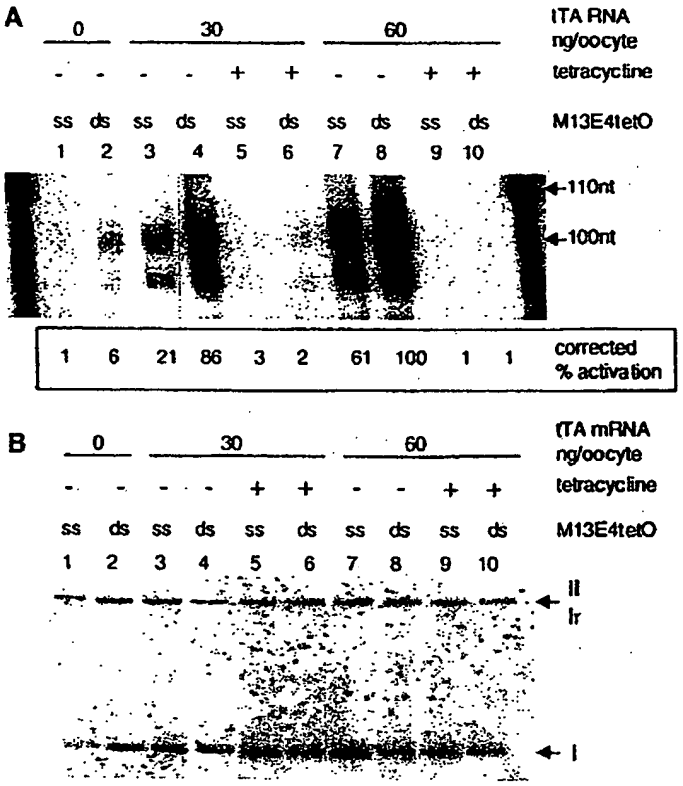

FIG. 5. Single-stranded strategy for tetracycline-regulated transcription. (A) Tetracycline-regulated E4 transcription. Transcription of the E4 gene from microinjected ss or ds M13E4tetO in *Xenopus* oocytes was detected using the reverse transcription assay described under Materials and Methods from oocyte extracts using the strategy illustrated in Fig. 2A. Either 5 ng/oocyte M13E4tetO alone (lanes 1 and 2) or in combination with 30 ng of tTA mRNA (lanes 3–6) or 60 ng of tTA mRNA (lanes 7–10) were microinjected into oocytes. Tetracycline (200 ng/ml) was added to the culture media in some cases (lanes 5, 6, 9, and 10). For each variable, 15 oocytes were lysed and the equivalent of 10 oocytes used for RNA extraction (A) and 5 oocytes for DNA extraction and supercoiling assay (B). Each lane represents mRNA extracted from 2 oocytes. The percentage activation is given for each lane corrected for amount of supercoiled template as shown in B. Marker at 110 nt is shown on right and left of gel. (B) Chromatin assembly on microinjected M13E4tetO templates. The supercoiling assay was used as a measure of chromatin assembly in the oocyte. Fifteen oocytes from each variable were pooled and lysed, with the equivalent of 10 oocytes used for RNA extraction as described above and 5 oocytes for DNA extraction and supercoiling assay. Lanes are the same as described for A. In each case, the efficiency of the microinjection was determined based on the amount of circular supercoiled DNA (I). The different forms of DNA are indicated as circular supercoiled (I), relaxed (Ir), and nicked (II).

DISCUSSION

In this report we define the optimal conditions for tetracycline-regulated gene expression in the *Xenopus* system. Information presented in Fig. 1A clearly shows that the tTA activator can be expressed to high levels in the oocyte. Importantly, the ability to assess the introduction of the tTA protein at the single-cell level in the *Xenopus* oocyte enabled us to determine that high levels of tTA (expressed from 75 ng of mRNA; Fig. 1A) can be introduced into an oocyte with no detectable deleterious effects on transcription (Fig. 1B). High levels of tTA protein can also be expressed in *Xenopus* embryos without gross developmental abnormalities (Fig. 3C). This is of interest since the tTA protein has been suggested to have toxic effects [17]. We also know that this tTA protein binds to the tetO sites since it activates luciferase expression 50-fold in the oocyte and over 200-fold in the developing embryo. The advantage of the tetR system over other activating systems such as hormone/receptor-driven systems [12, 29, 39] is the capacity to turn off activation, permitting gene activation or repression over a defined window, making it ideal for developmental applications and transgenics.

Refinements in restriction-mediated transgenic sys-

tems for *Xenopus* [5] allow overexpression of a particular gene product such as a kinase-deficient dominant-negative FGF receptor [6]. Application of tetracycline-regulated expression would improve its regulatory potential. During early stages of development, gene expression could be controlled by tetracycline injection. At later stages, when tadpoles or adults are feeding, tetracycline could be added to the food or water supply. In tetracycline-regulated mouse transgenics, a reverse rtTA induction system [18], where addition of tetracycline results in induction rather than shutoff, has overcome problems associated with the tTA system, where the half-life and clearance of the inducer are required for regulation (reviewed in [40]). This reverse system may prove to be a more effective alternative in the *Xenopus* embryo to maintain a prolonged shutoff of gene expression followed by a rapid burst of activation during a specific developmental stage or in a specific tissue. This burst of activation could be readily monitored by coupling the expression of the gene of interest to a fluorescent marker protein such as GFP.

Fluorescent markers have been used to track cellular processes by linking them to proteins and following their path during cell division or development. In such an approach, the LacI repressor fused to GFP was used to follow the localization of lactose operon operator sequences inserted into replication origin regions in *B. subtilis* [41]. An elegant series of experiments in yeast [19–21] make use of the tetracycline operator/repressor system to follow sister chromatid separation to identify factors involved in sister chromatid cohesion. The efficiency of expression and binding of tetR to its operon in *Xenopus* demonstrated in this report would facilitate the rapid application of such approaches to the study of nuclear dynamics in this higher eukaryotic system.

We are presently applying the tetracycline-regulated system to explore questions related to chromatin dynamics. Recent reports examining the action of the chromatin-remodeling machines in modifying chromatin suggest a tracking or sliding mechanism rather than the previous notion of nucleosome displacement [22, 42, 43]. In this context the binding of tetR has been used as a physical boundary to the mobility of nucleosomes during *in vitro Drosophila* chromatin assembly [22], lending further credibility to a sliding mechanism of action for CHRAC. We are using tetR as a barrier to chromatin assembly to plot the progress of bidirectional repair coupled chromatin assembly [44, 45] along DNA.

In conclusion, we have extended the application of tetracycline-regulated gene expression by defining the optimal parameters for its use in both the *Xenopus* oocyte and developing embryo. In both cases conditional expression of a gene of interest can be reliably and reproducibly achieved, adding to the already well-established usefulness of the *Xenopus* model system.

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Controlling transgene expression to study *Xenopus laevis* metamorphosis

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Sperm-mediated transgenesis of *Xenopus laevis* is the first application of genetic methodology to an amphibian. However, some transgenes are lethal when they are expressed constitutively. To study the influence of these genes on amphibian metamorphosis and to generate F_1 progeny from mature transgenic adults, these transgenes must be placed under the control of an inducible system so that they can be activated at specific times in development. We show that two well known binary inducible gene expression systems supplement transgenesis for the study of *X. laevis* metamorphosis, one system controlled by the progesterone analogue RU-486 and the other controlled by the tetracycline derivative doxycycline. By inducing a dominant negative form of the thyroid hormone receptor under the control of doxycycline specifically in the nervous system we have delimited the developmental periods within which thyroid hormone controls innervation of the developing limb from the spinal cord.

Xenopus laevis have been used as a model system for cell and developmental biology for decades. However, the "restriction enzyme-mediated integration" transgenesis method (1) that introduces genes into *X. laevis* embryos before first cleavage is the first genetic tool applied successfully to amphibians. Homologous and heterologous ubiquitous and tissue-specific constitutive promoters are expressed accurately in the F_0 embryos and tadpoles, and their expression patterns are transmitted faithfully to the next generations (2). We are interested in the genetic pathways that are controlled by thyroid hormone (TH) during amphibian metamorphosis. Two methods that are used to assess gene function during embryogenesis, mRNA injection into the fertilized egg and the injection of morpholino oligonucleotides, have limited use in the study of metamorphosis or any late developmental event. Tadpoles are not even competent to respond to TH until the second week after fertilization (3). A valuable adaptation of a genetic system is one where a gene of interest can be kept silent but then induced by a small molecule at an appropriate time and place in a specific cell type. For the study of metamorphosis and to generate F_1 progeny, this procedure has the advantage that embryogenesis can take place while the transgene is silent. Breeding sexually mature individuals with silent but inducible transgenes can be continued for generations.

In this study, we have demonstrated the value of two binary-inducible systems in transgenic *X. laevis*, the RU-486/mifepristone-inducible system (4, 5) and the tetracycline (Tet)-inducible system (6–8). The use of these inducible systems allows us to express the transgenes by using tissue-specific promoters. The progesterone analogue RU-486 is the ligand that binds a modified progesterone receptor ligand-binding domain fused to a GAL4 DNA-binding domain and a VP16 activation domain (GLVP) (4). A tissue-specific promoter drives this modified receptor that activates a transgene located on a second plasmid. This transgene of interest is cloned downstream of UAS containing four 17-bp GAL4-binding sites and a minimal promoter from the E1B major late promoter (4). We established a line of *X. laevis* that is transgenic for both plasmids, and we describe some of the features of this inducible system.

The Tet-inducible system was first described in 1992 (6). The Tet-off version requires the presence of the ligand doxycycline (Dox) to keep the transgene silent, a strategy that is less convenient for long-term rearing of tadpoles. Therefore, we have concentrated on the Tet-on system (7) in which gene expression is induced by addition of Dox. We used an improved Tet-on system (8) that has a very low baseline and a robust induction by doxycycline. We established a transgenic frog line that, when induced with Dox, expresses a dominant negative form of the TH receptor (TRDN) fused to GFP only in neural tissues. When this transgene is expressed constitutively in the nervous system the tadpoles never convert to leg swimming and die at the climax of metamorphosis as quadriplegics (9). We have induced the transgene expression during different windows of tadpole development, and the results suggest that TH is involved in both the formation of motor neurons and the peripheral innervation of the limb muscles.

DEVELOPMENTAL BIOLOGY

Materials and Methods

Plasmids. The plasmids CMV/GLVP (4) and UAS-E1b-LUC (5) were gifts from B. W. O'Malley (Baylor College of Medicine, Houston). pUHDrtTA2S-M2 was a gift from W. Hillen (University of Erlangen, Erlangen, Germany) (8). A 700-bp *Eco*RI+*Bam*HI fragment containing rtTA2S-M2 was isolated and the *Bam*HI site was blunted. This fragment was subcloned into the *Eco*RI and *Stu*I sites of pCS2+ (10). The resulting construct was cleaved with *Hin*dIII and *Not*I, yielding a 1-kb fragment containing rtTA2S-M2 with an simian virus 40 poly(A) end. This DNA was subcloned into NβT2+plasmid (2, 11) to obtain NβT/rtTA2S-M2. Similarly the *Hin*dIII+*Not*I fragment of pCarGFP2 (12) was replaced with the *Hin*dIII+*Not*I fragment from pCS2+rtTA2S-M2 to obtain pCar/rtTA2S-M2. The plasmid pUHC13-3 was a gift from H. Bujard (University of Heidelberg, Heidelberg) (6). The tetO promoter fragment from pUHC13–3 was PCR-amplified with appropriate primers and subcloned into the *Sal*I+*Hin*dIII sites of pCS2+GFP to obtain pCS2+(tetO)GFP. The *Hin*dIII+*Not*I fragment of pCS2+(tetO)GFP was replaced by the *Hin*dIII+*Not*I fragment from pCS TRDN/GFP (12) to obtain pCS2+(tetO)TRDN/GFP.

Dox Treatment. A 50 mg/ml stock solution of doxycycline hyclate (Dox; Sigma) was stored in the dark at −20°C. From 5 to 20 tadpoles were raised in 4 liters of 0.1× MMR (10 mM NaCl/0.2 mM KCl/0.1 mM $MgCl_2$/0.2 mM $CaCl_2$/0.5 mM Hepes, pH 7.5) with either 5 μg/ml or 50 μg/ml Dox. The medium was changed twice a week.

Immunohistochemistry. Immunohistochemistry with an α-phosphohistone 3 antibody has been described (13).

Abbreviations: TH, thyroid hormone; Tet, tetracycline; Dox, doxycycline; TRDN, dominant negative form of the TH receptor; NF, Nieuwkoop and Faber; NβT, neural-specific β-tubulin.

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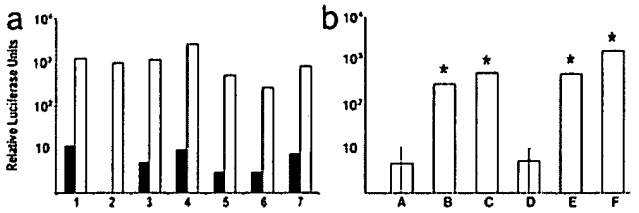

Fig. 1. The extent of inducibility by RU-486 of transgenes under the control of the modified progesterone system in *X. laevis* F_0 and F_1 tadpoles. (*a*) Luciferase reporter activity in the tail extracts of seven different F_0 tadpoles at stage 59 before (solid column) and 2 days after (open column) injection with 500 ng of RU-486/g of body weight. (*b*) Luciferase reporter activity in 1-week-old transgenic F_1 tadpoles. Column A, injected with 70% ethanol alone; column B, 24 h after a single injection of 5 ng of RU-486; column C, 24 h after a single injection of 50 ng of RU-486 in 70% EtOH; column D, uninduced; column E, induced with 10 nM RU-486 added to the tadpole rearing water for 3 days; and column F, induced with 100 nM RU-486 added to the tadpole rearing water for 3 days. In *b*, values are ± SEM and bars with * are significantly different ($P > 0.05$) relative to the uninduced transgenic and wild-type animals.

Luciferase Activity Assay and Protein Content. Two-millimeter pieces of tadpole tail were homogenized with a Tissuemizer in luciferase extraction buffer, the extracts were centrifuged at 22,400 × *g* for 5 min, and 50 μl of the supernatant was used to determine luciferase activity (14). The luciferase activity for each tail was normalized to total protein in the extract by using the Pierce protein measurement kit.

Results

The RU-486-Inducible System. The RU-486 system was tested in transgenic tadpoles in both F_0 animals and F_1 lines to examine the response to the inducer and its germ-line transmissibility. The two plasmids, CMV/GLVP (4) and UAS-E1b-LUC (5), were cotransformed into *X. laevis* by the restriction enzyme-mediated integration method (1), the tadpoles were raised to Nieuwkoop and Faber (NF) stage 59 and then injected with 500 ng of RU-486 per g of body weight. The up-regulation of the luciferase reporter in the tails of seven individual F_0 tadpoles (Fig. 1*a*) was at least one order of magnitude. In one case no detectable uninduced baseline level occurred. A sexually mature transgenic male was mated with a wild-type female frog. As determined by PCR, 52% of the F_1 progeny (14 of 27) were transgenic for both transgenes. As expected all the transgenic progeny had similar baseline levels, and the luciferase reporter was induced to the same extent (Fig. 1*b*). Even though RU-486 added to the rearing water does induce the transgene, its insolubility makes it difficult to control the amount that is being delivered and to determine the penetration of RU-486 to all tissues. In addition, it is not desirable to repeatedly inject the same animal. For these reasons, we decided to test the Tet method.

The Tet-Inducible System. To prepare Tet transgenic tadpoles, two plasmids were cotransformed by the restriction enzyme-mediated integration method. One has the ubiquitous simian cytomegalovirus (sCMV) promoter driving the modified Tet-binding protein (8). The second plasmid has Tet operator (TetO) elements upstream of GFP. One-week-old transgenic animals were induced with 5 μg/ml Dox added to the rearing water, and GFP expression was monitored at varying times (Fig. 2 *a* and *b*). The GFP was detected within 4 h and reached its peak expression 12 h after addition of Dox. The distribution of GFP expression is identical with that seen by using the same promoter driving GFP under constitutive control (2). To quantify the fold induction and measure the baseline expression a luciferase reporter

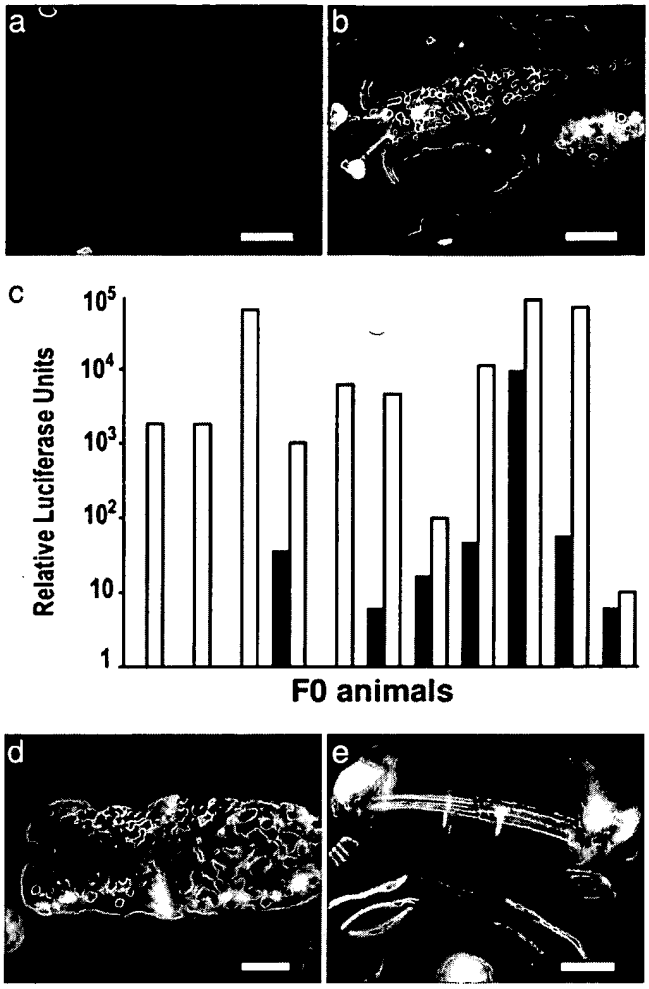

Fig. 2. Inducibility and tissue-specific expression of the Tet system in transgenic *Xenopus* F_0 and F_1 tadpoles. Expression of the GFP reporter gene in a 1-week-old F_0 transgenic animal before (*a*) and after (*b*) 12 h of treatment with 5 μg/ml Dox. (*c*) Luciferase activity in the tail extracts of 11 different F_0 transgenic animals before (solid bar) and after (open bar) induction with 5 μg/ml Dox for 48 h. Tissue-specific expression in 1-week-old tadpoles induced with 5 μg/ml Dox using the NβT (*d*) and cardiac actin (*e*) promoters driving inducible GFP. [Bars = 500 μm (*a*, *b*, and *e*) and 200 μm (*d*).]

was used instead of GFP. A plasmid containing the sCMV promoter regulating rtTA2S-M2 was cotransfected with one in which tetO drives luciferase. The F_0 tadpoles were grown to stage 56. About 2 mm of tail was removed from each tadpole before and after 24 h of 5 μg/ml Dox treatment, and extracts were assayed for luciferase activity and normalized for total protein content. The levels of reporter inducibility varied between individuals. However, in all but one animal the baseline of reporter gene activity was low or undetectable (Fig. 2*c*). Inducible levels varied from two to four orders of magnitude. To test the tissue specificity of this system, Dox-inducible GFP expression was placed under the control of two tissue-specific *X. laevis* promoters, neural-specific β-tubulin (NβT) (2) and the muscle-specific promoter (pCar) (1, 12). The Dox-induced expression patterns could not be distinguished from those of the constitutively expressed promoters (Fig. 2 *d* and *e*).

Inducing a Transgene Within Developmental Windows. When a dominant negative form of TH receptor α (TRDN) is expressed specifically in the developing brain and spinal cord, the tadpoles

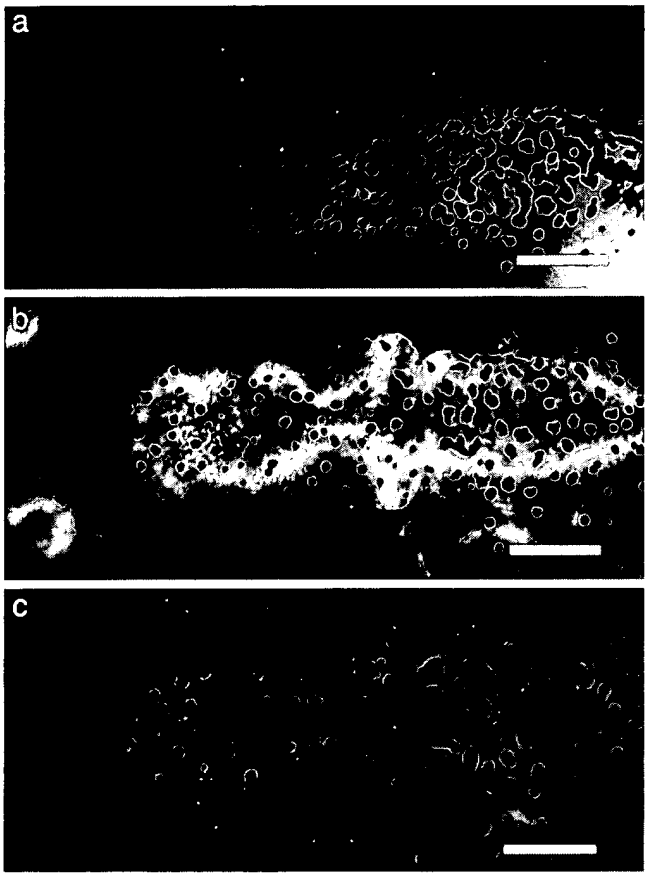

Fig. 3. Kinetics of induction in the brain of the TRDN/GFP fusion protein transgene driven by the NβT promoter. Dox (50 μg/ml) was added to the rearing water. The same animal before induction (*a*), after 12 h of Dox induction (*b*), and 24 h after withdrawal of Dox (*c*). (The left is anterior in all images.) (Bars = 500 μm.)

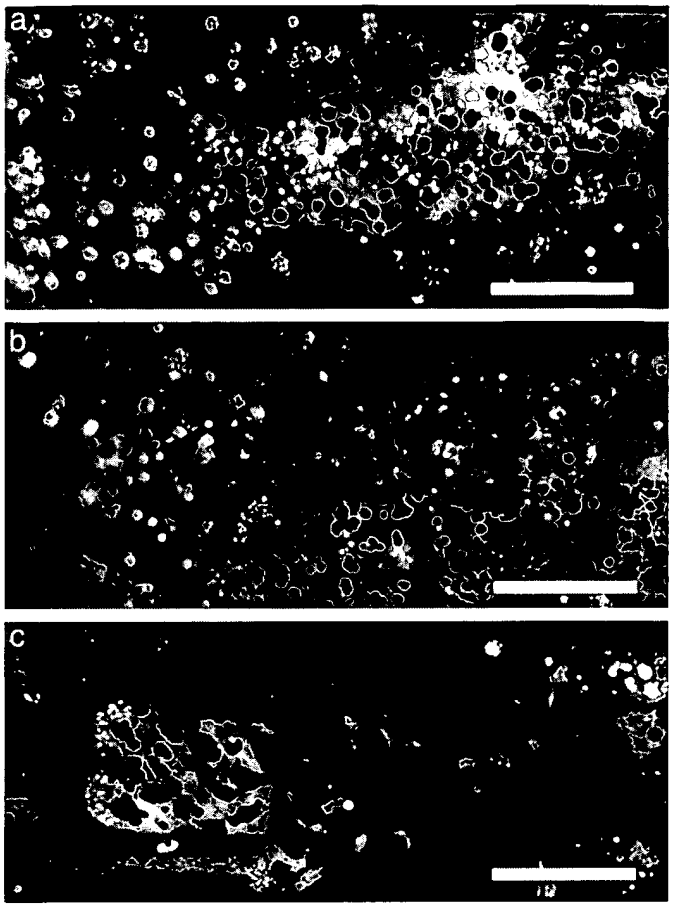

Fig. 4. TH-induced proliferation of the cells that line the brain ventricle was monitored by anti-PH3 staining (bright spots) in 1-week-old transgenic tadpoles after 4 days of treatment with 10 nM 3,5,3′-triiodothyronine (*a*), 10 nM 3,5,3′-triiodothyronine plus 50 μg/ml Dox (*b*), or untreated tadpoles (*c*). (The left is anterior in all images.) (Bars = 500 μm.)

cannot convert from tail to leg swimming at metamorphic climax (NF stage 60) (9). The most severe phenotype is complete paralysis of the fully developed limbs and death at metamorphic climax (9). Less severely affected animals have a delayed conversion to leg swimming at the climax of metamorphosis. This phenotype has its origins in the spinal cord where TH controls the formation of limb motor neurons (9). In previous experiments the constitutive NβT promoter expressed the TRDN transgene throughout embryonic and tadpole development in the entire nervous system. Because the severely affected animals die at the climax of metamorphosis it is impossible to obtain a breeding line. A Dox-inducible NβT/TRDN (fused to GFP) transgenic male frog was grown to sexual maturity and bred with a wild-type female, producing hundreds of F_1 tadpoles. This transgenic male has both transgenes inserted together into two different chromosomes because 75% of the progeny can be induced to express the TRDN-GFP reporter. The GFP-fused TRDN transgene was observed in F_1 progeny 4 h after addition of 50 μg/ml Dox to the rearing water (Fig. 3 *a* and *b*). The GFP/TRDN transgene product remained visible at a low level 24 h after removing Dox from the water (Fig. 3*c*) but could no longer be detected 48 h after Dox withdrawal (data not shown). The effectiveness of the NβT/TRDN transgene in F_1 animals was demonstrated by treating 1-week-old tadpoles with 50 μg/ml Dox and 10 nM 3,5,3′-triiodothyronine for 4 days. The TH-induced cell division that normally occurs in the cells lining the brain ventricle (13) is blocked by the transgene (Fig. 4). More than 50 uninduced transgenic tadpoles were grown to metamorphosis. None of them had even a mild leg-swimming phenotype. Tadpoles were sorted into control and transgenic groups during embryogenesis by their GFP expression after a 24-h induction with 50 μg/ml Dox. This early and brief 24-h induction of the transgene had no effect on development and metamorphosis. Transgenic tadpoles that were treated throughout tadpole life (from stage 46 to climax) all developed a strong phenotype (Table 1). The limb paralysis phenotype occurred in tadpoles treated with high levels of Dox from stage 46 to 52 when limb motor neuron progenitor cells are proliferating in the spinal cord. None of the tadpoles grown in the lower concentration of Dox (5 μg/ml) developed a severe phenotype. Transgenic tadpoles that were induced to express the transgenes as late as stage 56 developed the uncoordinated limb phenotype (Table 1). Tadpoles induced with Dox after stage 58 were normal.

Discussion

The most commonly used method to study gene function in *X. laevis* embryos, injection of mRNA into the fertilized egg, is not useful for the study of metamorphosis, which cannot be studied until the second week after fertilization. The first technique to address gene function in metamorphosis was the intramuscular injection of cloned genes directly into the tadpole tail (15). These genes are expressed in a mosaic manner in a limited number of tail muscle cells of living tadpoles. On the other hand, sperm-

Table 1. Summary of quadriplegic phenotypes in F_1 transgenic animals treated with different concentrations of Dox and stages

Developmental stages in Dox treatment	Dox dosage*	No. of tadpoles with phenotype†		
		No phenotype	Mild phenotype	Severe phenotype
46–52	High	1	3	1
46–59	High	0	0	3
52–59	Low	2	7	0
52/53–59	High	0	5	9
56–end	High	1	3	3
58–end	High	6	1	0
59–end	High	5	0	0

*High dosage is 50 μg/ml; low dosage is 5 μg/ml.
†Mild phenotype, leg swimming is delayed at climax; severe phenotype, severe paralysis, animals die without completing metamorphosis.

mediated (restriction enzyme-mediated integration) transgenesis (1) integrates a transgene into the genome before first cleavage. This method coupled with an inducible gene expression system that is controlled by a tissue-specific promoter provides a plausible way to direct transgene expression temporally and spatially and to establish lines of transgenic animals with lethal transgenes expressed in specific tissues. Our laboratory has previously assessed the value of the heat shock (2) and metallothionine (unpublished data) promoters, but both of them result in substantial baselines of reporter activity. The metallothionine promoter also has a tissue-specific expression bias (e.g., stronger expression in pronephros and pharynx).

In this article, we compared two different strategies for inducing transgene expression in transgenic *X. laevis*. Although RU-486 added to the rearing water up-regulated a ubiquitously expressed transgene, it was not tested for prolonged exposure, as was the Dox inducer for the Tet system. RU-486 is very insoluble in water so that controlling its delivery is a problem. Multiple intraperitoneal injections over a long period are detrimental to the tadpole's development and longevity. This method is suitable for experiments that require a single injection of the inducer. The very low baseline of transgene expression of the RU-486-inducible system (Fig. 1*b*) will make it useful for tissue ablation studies such as those using diphtheria toxin (16).

The second system we tested, the Tet-inducible system, has great advantages for use in conjunction with *Xenopus* transgenesis. Dox can be added throughout any window of development. The extent and specificity of transgene expression depends only on the fidelity of the promoter driving the Tet activator and the amount of the inducer. The ability to vary the level of transgene expression by changing the concentration of the inducer as tadpoles develop is another advantage of the inducible system. The highest dose of Dox that we used was 50 μg/ml in the rearing water. One-week-old tadpoles grew to metamorphosis in the continual presence of 50 μg/ml Dox. The animals eat, grow, and ultimately metamorphose normally under these conditions. The only abnormality observed after long-term exposure to a high concentration of Dox was a twisted tail due to a notochord defect. The lower dose of 5 μg/ml Dox induces the transgene to a lower level, causing the milder leg paralysis phenotype. This lower Dox concentration does not affect notochord morphology. With the Tet-inducible system driving the TRDN transgene, we analyzed the developmental times when TH controls limb innervation. We confirmed that premetamorphic expression of the transgene (up to NF stage 52; Table 1), a time when limb motor neurons are proliferating in the spinal cord (9), causes a mild phenotype. Likewise, application of the inducer beginning at NF stage 52 when the number of limb motor neurons is highest also results in the delayed leg swimming that is characteristic of this phenotype (9). By stage 56 nerves have entered the growing limbs and the number of limb-specific motor neurons has dropped to a fraction of the peak number reached at stage 52 (17, 18). However, the final clustering of the acetylcholine receptors in the limbs does not occur until about stage 58 (9). This experiment strongly indicates that at least two important TH-induced events are involved in the successful innervation of limbs, an early TH-controlled replication of ventricular cells in the spinal cord that give rise to limb motor neurons and then the final maturation of synapses between the nerves that enter the growing limbs and the muscle.

We have shown that transgenic *X. laevis* carrying a lethal transgene, controlled by an inducible system, can be grown to sexual maturity and will breed and transmit the transgene to F_1 progeny. The Tet system is particularly valuable for keeping a transgene silent and then inducing it within a precise window of development in a specific cell type. This methodology will have benefits for *Xenopus* research, in general, but it will be essential for genetic modifications that influence metamorphosis.

We thank our colleagues for comments and suggestions, Rejeanne Juste for expert technical assistance, and Drs. H. Bujard, W. Hillen, and B. W. O'Malley for their generous gifts of plasmids. This research was supported by grants from the National Institutes of Health and the G. Harold and Leila Y. Mathers Foundation (to D.D.B.).

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Generation of Heme Oxygenase-1-Transgenic Rats

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Heme oxygenase-1 (HO-1) expression protects cells from a variety of cellular insults and inhibits inflammation. However, its role in the regulation of immune responses has not yet been clearly established. We generated HO-1 transgenic rats to directly test the impact of HO-1 on the different immune mechanisms. To temporally control the expression of HO-1, we used a one-plasmid tetracycline (tet)-inducible system. This plasmid contains the H-2K^b promoter, which transcribes the tet transactivator (tTA) and expression of a human HO-1 cDNA is obtained in the absence of tetracycline. The DNA construct was microinjected into one-cell rat embryos and mothers and pups were maintained with tetracycline. Eight transgenic founders were obtained. Analysis of transgene expression in the absence of tet showed that 2 lines (12.4 and 12.6) expressed HO-1 mRNA in several organs (as detected by reverse transcription polymerase chain reaction) and at the protein level only in the thymus. Expression levels of transgene-derived HO-1 increased after withdrawal of tet compared with transgenic rats maintained with tet, as detected by analysis of mRNA levels by quantitative real-time reverse transcription polymerase chain reaction. Gross examination and histopathological analysis of several organs in both lines showed no anomalies. Thymocytes and splenocytes of both lines showed normal cell subpopulations and allogeneic proliferation compared with controls. Systemic immune responses against cognate antigens were normal in both lines, as evaluated by the proliferation of lymph node cells and the production of antibodies against keyhole limpet hemocyanin after immunization. Animals from line 12.6 rejected transplanted allogeneic hearts with the same kinetics as controls. In conclusion, short-term induction of HO-1 overexpression did not modify immune responses compared to those of control non-transgenic animals. Exp Biol Med 228:466-471, 2003

Key words: HO-1; transgenic rats; immune response; transplantation

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the oxidative degradation of heme into biliverdin, free iron, and carbon monoxide. This 32-kDa stress-inducible protein provides protection against a variety of cellular injuries, such as oxidative stress, proinflammatory cytokines, and proapoptotic inducers (1-3). HO-1 has been shown to have anti-inflammatory actions (2), to suppress macrophage activation (4), to mediate the anti-inflammatory effects of interleukin-10 (5), and to inhibit allograft and xenograft rejection (2, 6, 7). However, whether HO-1 modulates antigen-specific immune responses or other lymphocyte functions has not yet been established. Previous publications describing transgenic mice for HO-1 have used promoters that are specific for neurons (8), vascular smooth muscle cells (9), lung cells (10), or cardiomyocytes (11, 12).

The aim of this study was to generate HO-1-transgenic rats to directly analyze the impact of HO-1 on various immune functions. We used the ubiquitous H-2K^b promoter to target HO-1 expression primarily to endothelial cells and leukocytes (13) and the tet-off inducible system (expression in the absence of tet) to control transgene expression. We obtained two lines of transgenic rats that, after 10 days of tet withdrawal, overexpressed human HO-1 mRNA in all organs. Transgene-derived HO-1 was detectable at the protein level only in the thymus. Thymocytes from transgenic rats displayed cell subsets and allogeneic proliferative responses *in vitro* comparable to those of nontransgenic animals. HO-1 transgenic rats used as recipients rejected cardiac allografts with the same kinetics as control rats. After immunization, transgenic rats showed normal antibody and proliferative responses against cognate antigens.

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Materials and Methods

DNA Construct. The backbone of the construct (pCombi) used to generate transgenic rats with tet controllable expression was kindly provided by Dr. U. Certa (Hoffmann-La Roche, Basel, Switzerland) and has been used previously to generate transgenic mice (14). In the construct used to generate HO-1 transgenic rats (pCombiHO-1; Fig. 1), the mouse ubiquitous H-2K^b promoter linked to an enhancer from the murine T cell receptor α chain gene (13) and a hybrid intron were inserted 5′ of the coding sequence for the tTA and SV40 polyadenylation (polyA) sequences of pCombi. In the absence of tet, the tTA binds to the tet operator (tetOP) and, in conjunction with a CMV minimal promoter (pCMVmin), activates transcription of an expression cassette containing (from the 5′ end to the 3′ end) a β globin intron, the human HO-1 cDNA (kindly provided by S. Shibahara, Tohoku University School of Medicine, Sendai, Japan) in which a FLAG sequence was inserted in its 3′ end, and SV40 polyA sequences.

Generation of HO-1 Transgenic Rats. Transgenic rats were generated by pronuclear microinjection of Sprague-Dawley eggs (IFFA-CREDO, L'Arbresle, France) with the *SalI-Bam*HI fragment of pCombiHO-1 (9 kb) using procedures previously described (15, 16). To inhibit transgene expression, tet (1 mg/ml) and sucrose (36 mg/ml) were included in the drinking water (protected from light) of surrogate mothers and litters derived from microinjection. Analysis of transgene expression was performed at least 10 days after tet withdrawal. Transgenic rats were first identified by analysis of tail DNA by polymerase chain reaction (PCR) using human HO-1 primers: sense; 5′-GTC TTC GCC CCT GTC TAC TT-3′ and antisense; 5′-CTC TTC TAT CAC CCT CTG CCT-3′. PCR was also performed in parallel for the rat reference gene hypoxanthine phosphoribosyltransferase using the following primers: sense; 5′-GCG AAA GTG GAA AAG CCA AGT-3′; antisense; 5′-GCC ACA TCA ACA GGA CTC TTG TAG-3′. Both PCR reactions consisted of 5 min at 94℃, 5 min at 62℃ and 30 cycles of 72℃ for 30 sec, 60℃ for 30 sec and 94℃ for 30 sec. Positive animals were then confirmed by Southern blot analysis of DNA digested with *Eco*RV using a ^{32}P-labeled dCTP fragment of pCombiHO-1. Determination of zygosity in HO-1 transgenic animals was performed using a new real-time PCR-based method (17) using an ABI Prism 7700 Sequence Detector System (PE Applied Biosystems, Foster City, CA).

RNA Extraction and Real-Time Quantitative PCR. Total RNA was isolated using Trizol (Life Technologies, Paris, France), treated with DNAse (Roche, Indianapolis, IN) and reversed transcribed (Life Technologies, Paris, France) into cDNA. Real-time quantitative PCR was performed using primers for human HO-1: forward; CTC AAC ATC CAG CTC TTT GAG GAG TTG CAG G-3′ and reverse; 5′-TGG GAG CGG GTG TTG AGT-3′ and a labeled TaqMan® probe 5′FAM-CTC AAC ATC CAG CTC TTT GAG GAG TTG CAG G-TAMRA3′. Rat hypoxanthine phosphoribosyltransferase was amplified using the same primers described above and a labeled TaqMan® probe 5′VIC-CAA AGC CTA AAA GAC AGC GGC AAG TTG AAT-TAMRA3′. Transcript levels were calculated according to the $2^{-\Delta\Delta Ct}$ method (18).

Analysis of HO-1 Expression. Immunohistological analysis was performed on cryostat sections using a rabbit anti-HO-1 antibody (reacting with both human and rat HO-1) (Stressgen, Victoria, British Columbia, Canada) using techniques previously described (7). HO-1 enzymatic activity was analyzed in the microsomal fraction of the thymus as previously described (19).

Keyhole Limpet Hemocyanin (KLH) Immunization. KLH (Sigma, St Louis, MO) was injected in the footpad as previously described (20). Anti-KLH antibodies were detected in sera using an enzyme-linked immunosorbent assay and proliferative responses against KLH were analyzed using popliteal lymph node cells 10 days after immunization as previously described (20).

Mixed Leukocyte Reaction (MLR). Splenic or thymic cells from non-transgenic or HO-1 transgenic rats (MHC haplotype RT1^u) were cultured with γ-irradiated allogeneic antigen presenting cells (APCs) from LEW.1A rats (MHC haplotype RT1^a) with or without recombinant IL-2 (100 U/ml) as previously described (20).

Cytofluorimetry. Cells were incubated with mAbs for 20 min at 4°C, washed twice, and analyzed using a

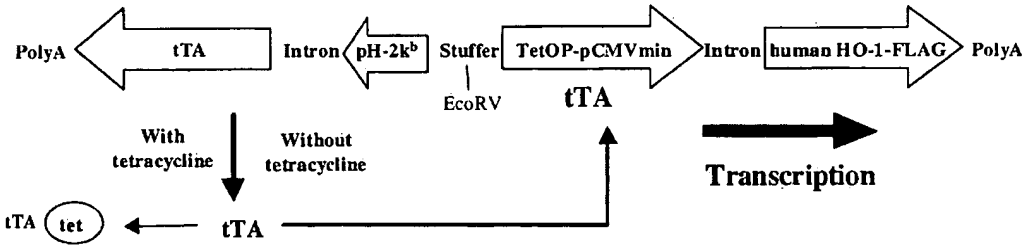

Figure 1. pCombiHO-1 DNA construct used for the generation of HO-1 transgenic rats. The ubiquitous H-2K^b promoter drives the expression of the tet transactivator (tTA). In the absence of tetracycline (tet), tTA binds to the tet operator (tetOP) and with a CMV minimal promoter (pCMVmin), activates transcription of an expression cassette containing a β globin intron, the human HO-1 cDNA with a FLAG sequence in its 3′ end and SV40 polyA sequences.

FACScalibur (Becton Dickinson, Mountain View, CA). The following mouse anti-rat mAbs obtained from the European Collection of Cell Culture (Salisbury, UK) were used after coupling to FITC, biotin, or PE (Bioatlantic, Nantes, France): OX6 (MHC class II, B cells, macrophages and activated T cells), W3/25 (CD4; mainly CD4+ T cells), OX7 (Thy-1.1; CD90, thymocytes), OX33 (CD45RB; B cells), OX42 (CD11b and CD11c; macrophages and dendritic cells), OX8 (CD8; CD8α + T cells), OX39 (CD25; activated T cells), OX22 (CD45RC), and OX85 (CD62L; naive T cells). The JJ319 MAb (CD28; T cells) was kindly provided by Dr. T. Hüning (University of Würzburg, Germany). The FITC-conjugated anti-CD3 (clone G4.18; T cells), anti-B7-2 (CD86; macrophages and dendritic cells), and PE-conjugated anti-B7.1 (CD80; macrophages and dendritic cells) mAbs were purchased from PharMingen (San Diego, CA). The FITC anti-CD161a (NKR-P1A, NK cells) was purchased from Serotec (Oxford, UK).

Heart Transplantation. Cardiac allografts from LEW.1A donors were placed into the abdomen of HO-1-transgenic rats and graft survival was monitored daily by palpation through the abdominal wall. Rejection was defined as cessation of cardiac beating.

Histopathological Analyses. Hematoxylin and eosin-stained tissue sections of paraffin-embedded samples of the indicated organs were analyzed by an experienced pathologist (C.T.).

Results

Generation of HO-1 Transgenic Rats with the Tet-Off System. After transient transfection of COS cells with pCombiHO-1, expression of HO-1 was undetectable in the presence of tet (2 μg/ml) and was induced in its absence, as analyzed by Western blot and enzymatic assays in cellular lysates (data not shown).

Immediately after microinjection of the 9-kb fragment of pCombiHO-1, 702 one-cell embryos were transferred into foster mothers and 111 pups were obtained. Eight founders (7.2% of newborns) were identified by PCR analysis and confirmed by Southern blot analysis as carriers of the entire HO-1 transgene integrated in a single-site (data not shown). The frequency of transgenic rats obtained with pCombiHO-1 was comparable to that obtained previously for other transgenes (16, 21, 22). Four founders transmitted the transgene to their descendants and transgenic lines were derived from each of them.

Analysis of Transgene-Derived HO-1 mRNA. Expression of transgene-derived HO-1 mRNA was analyzed using real-time reverse transcription PCR in organs from rats continuously kept with tet or withdrawn from tet for 10 days. Two lines among 4, 12.4 (Fig. 2A) and 12.6 (Fig. 2B), expressed transgene-derived HO-1 mRNA, mainly in lymphoid organs and the highest levels being observed in the thymus, as previously described in transgenic mice generated with the H-2K^b promoter (13). HO-1 mRNA increased in both lines in the absence of tet. Line 12.4 expressed higher levels in the absence and lower levels in the presence of tet than line 12.6. Low levels of HO-1 mRNA were observed in the heart and kidney in line 12.4 but not in line 12.6. The liver did not show HO-1 mRNA accumulation in neither line.

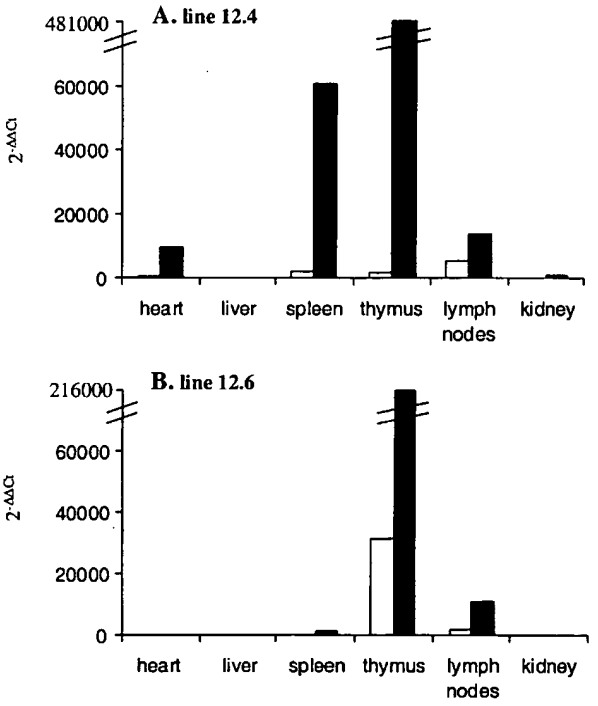

Figure 2. Quantification of hHO-1 mRNA. Real-time quantitative RT-PCR was used to analyze hHO-1 in the indicated organs from (A) line 12.4 and (B) line 12.6 kept with or without tetracycline (tet) for 10 days. Results from one animal in each condition are representative of two to three animals analyzed in each line.

Analysis of Transgene-Derived HO-1 Protein Expression. Homozygous HO-1 transgenic rats from lines 12.4 and 12.6 were continuously kept with tet or withdrawn from tet for 10 days and their organs were analyzed for HO-1 protein expression. As observed by immunohistological analysis (Fig. 3), low levels of endogenous HO-1 were detected in the thymus of control rats and lines 12.4 and 12.6 expressed higher levels. Cells expressing HO-1 in transgenic rats were scattered in the cortex and medulla. Higher expression of HO-1 was confirmed by Western blot analysis in the thymuses of HO-1 transgenic rats versus controls (data not shown).

Analysis of HO-1 enzymatic activity showed increased levels in the thymus in lines 12.6 and 12.4 versus controls (2 and 1.49, respectively vs 0.3 nmol/mg/h). Transgene-derived HO-1 was undetectable in the lymph nodes, spleens, livers, kidneys, and hearts of these animals, as analyzed by immunohistology and Western blot (data not shown).

Thymic Cell Subsets and Immune Responses Were Unmodified in HO-1-Transgenic Rats. Histopathological analysis of the thymus, lymph nodes, spleen, liver, kidney, intestine, lung, and heart in lines 12.4 and 12.6 after tet withdrawal revealed no anomalies (data not shown). Analysis by cytofluorimetry of the major leukocyte subsets (CD4+, CD8+, B cells, macrophages, CD90+ thymocytes, and NK cells) of lines 12.6 (Fig. 4A) and 12.4 (data not shown) revealed no differences compared with controls of the same age. Other markers, such as CD25, CD45RC, CD62L, CD86, and CD80, did not reveal any differences in the thymic cells of transgenic versus nontransgenic rats. The same analysis performed on spleen cells from transgenic lines 12.4 and 12.6 did not show any phenotypic differences compared with controls (data not shown).

Analysis of allogeneic proliferation of thymic cells from transgenic rats (haplotype $RT1^u$) against APCs from LEW.1A (haplotype $RT1^a$) rats showed strong proliferation for lines 12.6 (Fig. 4B) and 12.4 (data not shown), which were comparable to those of control nontransgenic rats. The same MLRs performed with spleen cells from transgenic lines 12.4 and 12.6 did not show any differences compared with controls (data not shown).

To evaluate the immune responses of transgenic rats against cognate antigens, the animals were continuously kept with tet or withdrawn from tet for 10 days. KLH was subsequently injected into the footpad and 10 days later the analyses of proliferative responses of draining lymph nodes cells (Fig. 4C) and sera anti-KLH antibody levels (Fig. 4D) in HO-1-transgenic rats showed responses similar to those of controls.

Allograft Survival Was Not Prolonged in HO-1-Transgenic Rats. We used transgenic rats of both lines as recipients of cardiac allografts from LEW.1A donors. Rejection of LEW.1A heart by non-transgenic controls (6.2 ± 0.8, $n = 3$) was indistinguishable from those of transgenic recipients (line 12.6, 6 days, $n = 3$; line 12.4, 7 days, $n = 3$).

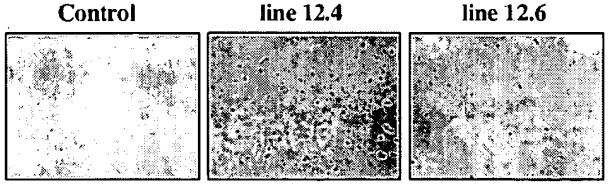

Figure 3. Thymic HO-1 expression. Homozygous HO-1 transgenic rats were maintained without tet for 10 days and HO-1 expression was analyzed on thymus cryostat section by immunohistology using an anti-HO-1 antibody (objective 20). Results from one animal for each condition are representative of two to three animals analyzed in each line.

Discussion

We generated rats transgenic for HO-1, which overexpressed transgene-derived HO-1 protein in the thymus. The $H\text{-}2K^b$ promoter was used because of the fact that in transgenic mice, it has been shown to drive expression of transgenes primarily in leukocytes and endothelial cells (13). Overall, the expression levels of HO-1 obtained in the transgenic rats was low because its expression was only detectable in two of four lines and transgene-derived HO-1 protein was only detectable in the thymus. Although transgenic pigs have also been generated using the same promoter (23), we cannot formally exclude the possibility that species restrictions may exist in the rat for high expression of transgenes placed under its transcriptional control. Alternatively, as is the case for every promoter lacking insulating sequences, its activity may be largely dependent on the chromatin configuration at the point of insertion of the transgene into the genome.

The tet-off system integrated into a single DNA construct used in this study has been previously applied to generate lines of transgenic mice, from which a proportion showed tet-controlled expression of the transgene (14). Both rat transgenic lines showed higher HO-1 expression in the thymus in the absence than in the presence of tet at the mRNA level suggesting regulation of HO-1 expression.

Among other observable protective effects, mice transgenic for HO-1 have shown decreased inflammation after oxidative injury (8, 10–12), but the effect of HO-1 in other inflammatory models has not been analyzed in transgenic animals. In our study, rats transgenic for HO-1 displayed thymic and splenic cell subsets as well as cellular and humoral anti-KLH responses comparable with those of nontransgenic rats. In MLRs, thymocytes and splenocytes from HO-1 transgenic rats showed proliferative responses of the same magnitude as control rats. Moreover, in a cardiac allograft model, HO-1 transgenic animals rejected allogeneic hearts with the same kinetics as controls. These results suggest that rats transgenic for HO-1 show normal differentiation of immune cells and immune responses after a relative short-term (10 d) induction of HO-1 expression. Neverthe-

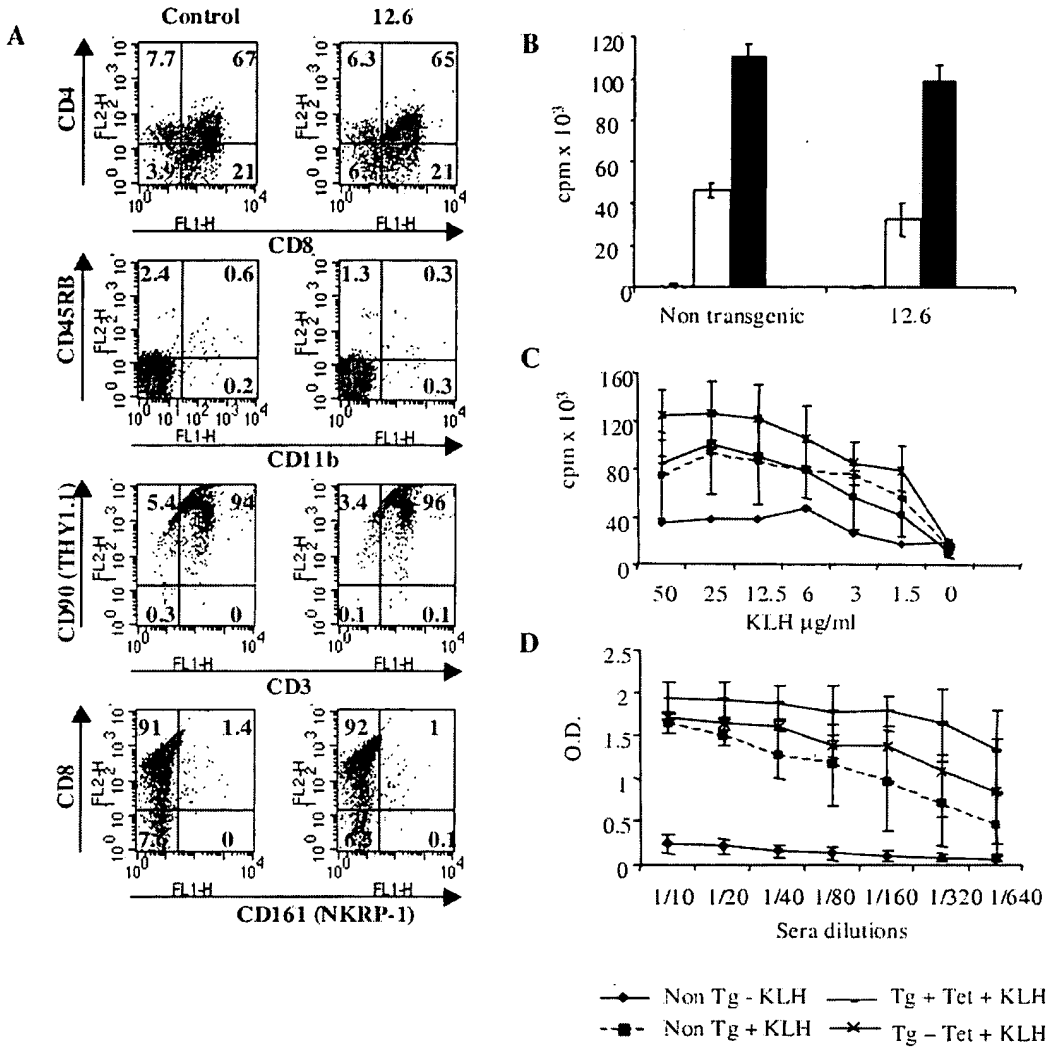

Figure 4. Thymic subsets and immune responses. Nontransgenic litter mates or homozygous HO-1 transgenic rats from line 12.6 maintained with or without tet for 10 days were analyzed for phenotype and function of their immune cells. (A) Thymic cells analyzed by cytofluorimetry. Numbers within each window represent the percentage of positive cells. (B) Proliferation of thymic cells cultured with medium alone (stippled histogram) or with allogeneic APCs (LEW.1A) in the absence (white histogram) or presence (black histogram) of IL-2 (100 U/ml). [^{3}H] thymidine incorporation (mean ± SD of triplicate cultures) was evaluated after 5 days of culture. Nontransgenic (Non Tg) or transgenic (Tg) rats of line 12.6 were kept with or without tet (+ Tet or –Tet, respectively) for 10 days, immunized or not with KLH in the footpad and analyzed 10 days later. (C) Draining lymph node cells were removed and proliferation against different concentrations of KLH was tested *in vitro*. [^{3}H] thymidine incorporation (mean ± SD of triplicate cultures) was evaluated after 3 days of culture. Each curve represents the proliferation of cells from a single animal. (D) Anti-KLH antibodies were detected in sera of rats of line 12.6 using an ELISA. The results for the KLH experiments were an average of two animals from each condition. Results are expressed as optical density (OD).

less, the low HO-1 expression observed in secondary lymphoid organs do not allow us to conclude that HO-1 overexpression does not affect immune responses. New transgenic animals with high HO-1 expression in peripheral lymphoid organs are currently being generated. Thymic cells displayed clear HO-1 protein overexpression, and some important cell processes (apoptosis, cytokine production) have not yet been analyzed. Furthermore, overexpression during the whole life of these transgenic rats may modify thymocyte differentiation. The generation of transgenic rodents with expression of HO-1 by cells of the immune system will represent an important tool for the analysis of HO-1 function.

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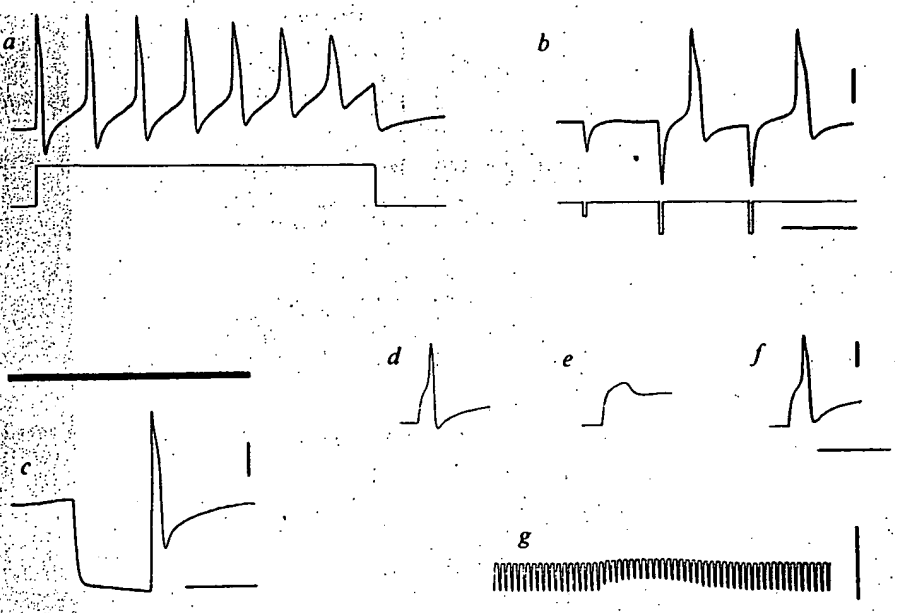

Fig. 2 Intracellular recordings from salivary gland cells of *H. ghilianii*. *a*, *b*, Action potentials, which may exceed 90 mV, are initiated by depolarizing current (*a*) and also following hyperpolarization by inward current (*b*; 100-ms pulses were applied). *c*, A hyperpolarizing current pulse in one salivary cell (lower trace) produces rebound excitation but there is no sign of electrical responses in an adjacent cell, recorded simultaneously at high gain on the upper trace. Experiments of this type indicate that the gland cells are not electrically coupled. *d*–*f*, Effect of 5 mM Co^{2+} added to the bathing solution. *d*, Control impulse in response to depolarizing current; *e*, 5 min after addition of $CoCl_2$ the action potential is abolished (a delayed rectification is apparent); *f*, recovery. This indicates that Ca^{2+} is the major current carrier for generation of action potentials. *g*, Brief application of 10^{-5} M serotonin produces a depolarization and increase in membrane conductance (indicated by reduction in amplitude of constant-current hyperpolarizing pulses). Voltage scales (vertical bars), 25 mV (1 mV in *c*, upper trace); time scales (horizontal bars), 2 s.

The natural stimulus for action potential generation, whether neural and/or hormonal, is unknown. Several putative neurotransmitters, however, (dopamine, serotonin and acetylcholine) were found to depolarize the gland cells, with an accompanying decrease in membrane resistance (Fig. 2*g*) and occasionally the production of no more than four impulses. Interestingly, in the presence of dopamine, applied depolarizing current was sometimes found to produce repetitive firing which was not simply a consequence of the depolarization produced by the drug. If the impulse provides a trigger for secretion, it seems unusual that the cells are normally so difficult to activate. Feeding, however, occurs very infrequently (every few months) and an action such as that of dopamine may mimic a natural process of bringing the gland into secreting condition.

In mammals, salivary and other exocrine gland cells are electrically inexcitable, producing graded potential changes (often hyperpolarizations) which may or may not be related to secretory function[10]. The *H. ghilianii* salivary cells are similar in their electrical excitability to mammalian endocrine cells such as those in the pancreas[12], adenohypophysis[13] and adrenal gland[14] (some molluscan exocrine glands produce action potentials[15]). This similarity extends to the anode-break excitation[8] shown by chromaffin[14] and anterior pituitary cells[13].

We have also found the *H. ghilianii* salivary gland to be suitable for molecular genetics because the cells have a very large ramifying nucleus that displays gene amplification of $\sim 10^6$ times; this should allow precise questions to be asked about the relationship between secretion and transcription/translation of identified genes. Thus, the *H. ghilianii* salivary gland, with its unusual combination of properties, represents a simple, accessible preparation with distinct experimental advantages for cellular studies of glandular secretion.

This specialized leech has been generally unavailable because in its natural habitat it is restricted to Amazonia. We have developed techniques for breeding this species and a facility has been set up by Biopharm to supply hementin to interested researchers.

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Production of transgenic rabbits, sheep and pigs by microinjection

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Direct microinjection has been used to introduce foreign DNA into a number of terminally differentiated cell types as well as embryos of several species including sea urchin[1], *Candida elegans*[2], *Xenopus*[3], *Drosophila*[4,5] and mice[6–11]. Various genes have been successfully introduced into mice including constructs consisting of the mouse metallothionein-I (MT) promoter/regulator region fused to either the rat or human growth hormone (hGH) structural genes. Transgenic mice harbouring such genes commonly exhibit high, metal-inducible levels of the fusion messenger RNA in several organs, substantial quantities of the foreign growth hormone in serum and enhanced growth[12,13]. In addition, the gene is stably incorporated into the germ line, making the phenotype heritable. Because of the scientific importance and potential economic value of transgenic livestock containing foreign genes, we initiated studies on large animals by microinjecting the fusion gene, *MT–hGH*[13], into the pronuclei or nuclei of eggs from superovulated rabbits, sheep and pigs. We report here integration of the gene in all three species and expression of the gene in transgenic rabbits and pigs.

Studies with mouse ova indicated that integration of a gene into host chromosomes is much more efficient with nuclear than with cytoplasmic injection[14]. On this basis, we reasoned that nuclear injection would be an appropriate first approach with other species. The first problem encountered was visualization

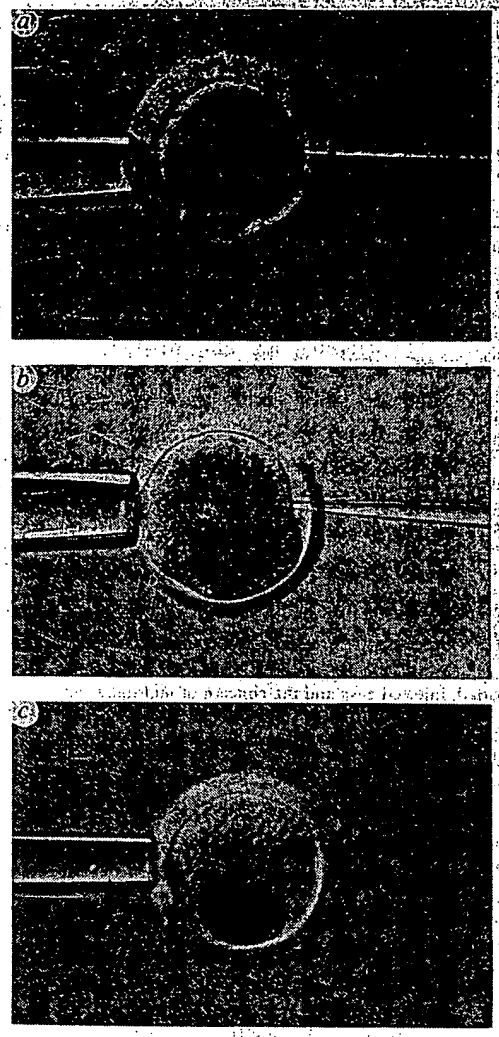

Fig. 1 Interference-contrast photomicrographs of one-cell fertilized ova from rabbit (*a*), sheep (*b*) and pig (*c*) following microinjection. Ova are held by a blunt holding pipette (diameter ~50 μm); an injecting pipette (diameter ~1.5 μm) has penetrated the zona pellucida, plasma membrane and pronuclear envelope. The tip is seen within the nucleoplasm immediately following injection of buffer containing DNA. The porcine ova (*c*) has been centrifuged at 15,000*g* for 3 min to reveal the normally obscure pronuclei[15]. Visualization of nuclear structures is aided by the use of interference-contrast optics, and microinjection is carried out under ×250 magnification using a Leitz microinjecting apparatus[10,14]. Injection was monitored by observing the diameter of the pronuclus or nucleus, which was expanded ~50%.

of the pronuclei or nuclei in the ova. Rabbit nuclear structures are readily seen (Fig. 1*a*). However, pronuclei and nuclei in sheep ova are difficult to locate and can only be seen by fluorescent microscopy using DNA specific fluorochromes (Hoechst, 33258) or by interference contrast (IC) microscopy (Fig. 1*b*). The combination of stain and ultraviolet light is damaging to the ovum (data not shown), so we used IC microscopy for microinjection. Fluorescent analysis indicated that IC microscopy is an effective method for pronuclear localization in approximately 80% of fertilized sheep eggs. Pig ova are opaque and no nuclear structures can be seen even with IC microscopy, but we found that centrifugation of pig ova at 15,000*g* for 3 min stratifies the cytoplasm (Fig. 1*c*), leaving the pronuclei or nuclei visible[15].

Once the nuclei could be visualized, microinjection was performed as described previously[10,14]. A few hundred copies of a 2.6 kilobase (kb) linear fragment containing the *MT-hGH* gene (see Fig. 2) were injected. Approximately 5,000 ova were injected and subsequently transferred to foster animals; about 500 of these resulted in fetuses or neonates (Table 1). The frequency of *MT-GH* integration was similar in the rabbit (12.8%) and the pig (11.0%) and low in sheep (1.3%). These integration efficiencies are probably accurate for the techniques being used because they are based on a large number of animals. The reasons for the lower integration frequency in these species compared to the mouse where it is ~27% are unknown but could be related to factors such as the concentration of DNA, buffer composition, age of the ovum and the structure of the chromosomes[14].

The number of copies of the *MT-hGH* gene that integrated was estimated by quantitative dot hybridization. Figure 2*d* shows the quantitation method as applied to transgenic pigs. Gene copy numbers ranged from 1 to 490 copies per cell (Table 2). The DNA from some of the transgenic animals was also analysed by restriction enzyme digestion of the chromosomal DNA followed by agarose gel electrophoresis and Southern blotting. Figure 2*a* shows the results obtained when the DNA was restricted with *Eco*RI, an enzyme that cuts once within the injected DNA. The probe detects two prominent bands in several rabbits and pigs. One band is close to the length of the injected DNA fragment (2.6 kb) and probably represents a tandem, head-to-tail array of the *MT-hGH* genes as is typically observed in transgenic mice[10,12]. The other band is approximately twice that length and might represent a head-to-head dimer, but further analysis will be required to test that possibility. When the DNA was restricted with *Sst*I (Fig. 2*b*), an enzyme that cuts twice within the injected DNA, two bands of the expected size were observed in all of the pigs and rabbits. Analysis of the sheep sample with *Eco*RI (not shown) and *Sst*I (Fig. 2*c*) revealed bands that were inconsistent with an intact *MT-hGH* gene, suggesting that the DNA had been trimmed or rearranged prior to integration.

Expression of the integrated genes was examined by quantitating *MT-hGH* mRNA by solution hybridization (Table 2). Only 4 of 16 rabbits analysed had any detectable *MT-hGH* mRNA in the liver, but the level was substantial in one of these. In mice, the frequency of expression of this gene is close to 70% (ref. 13). In pigs, mRNA levels were measured only in tail or ear samples because we did not want to risk adverse consequences of liver biopsy. Although tail and ear tissues are not primary sites of *MT* gene expression, we detected low levels of *MT-hGH* mRNA in several of the transgenic pigs (Table 2).

Plasma samples taken from pigs at birth and ~1 month later were analysed for hGH by radioimmunoassay. At birth, 11 of 18 pigs had detectable levels of hGH, ranging between 2 and 730 ng ml^{-1} (Table 2). One month later, hGH exceeded 300 ng ml^{-1} in three pigs. One rabbit also had a high level of hGH. Serum hGH as high as 64,000 ng ml^{-1} has been detected in transgenic mice, but accelerated growth rate was observed at levels of 20 to 80 ng ml^{-1} (ref. 13). None of these animals were exposed to high levels of zinc, a treatment that has been shown to activate *MT-hGH* gene expression ~10-fold in mice[13].

The effects of hGH on the growth of rabbits cannot be evaluated at present because only one live rabbit had detectable serum hGH and unfortunately it had malocclusion that impaired normal food consumption. Early indications are that the levels of hGH found in these transgenic pigs do not increase body weight dramatically. This may not be surprising considering that daily injections of bacterially synthesized hGH had no effect[16], and exogenous, highly purified porcine GH only stimulated growth by 10% when delivered during the major growth phase of the pig[17]. Transgenic offspring and littermate controls will need to be raised on normal and zinc-supplemented diets to determine precisely the effects of hGH on growth rate and other nutritional as well as endocrine parameters.

These experiments demonstrate that foreign genes can be introduced into several large animal species by microinjection of ova. Furthermore, expression of MT-hGH was obtained in rabbits and pigs. We used a fusion gene that has worked well in mice to demonstrate the feasibility of such techniques, and we are now trying several modifications in an effort to improve the level of expression and physiological response.

Table 1 Efficiency of producing *MT-hGH* transgenic rabbits, sheep and pigs by microinjection

Species	Transferred injected ova	Recipients	Integration frequency (%)	Expression frequency *MT-hGH* mRNA	Expression frequency Serum or plasma hGH
Rabbit*	1,907	73	28/218 (12.8)	4/16	1/1
Sheep†	1,032	192	1/73 (1.3)	ND	ND
Pig‡	2,035	64	20/192 (10.4)	11/20	11/18

Integration frequency is the number of animals (fetuses, stillborns and neonates) that retained the injected DNA/total number of animals resulting from injected ova. Six gilts bearing only injected eggs farrowed, producing 52 neonates, 5 of which retained DNA. In 31 gilts that farrowed, 204 fertilized control ova were transferred along with 859 injected ova to ensure sufficient embryos at implantation to maintain pregnancy. If survival of injected eggs to fetuses (16.4%) was similar for both groups, then injected eggs resulted in 140 of 252 fetuses and piglets produced, 15 of which retained injected DNA. We combined the data from the two groups to estimate integration efficiency. Expression frequency is the number of fetuses or neonates containing *MT-hGH* mRNA or plasma hGH per total number of animals examined. ND, not determined.

* Fertilized one-cell rabbit ova were flushed from the oviducts of superovulated New Zealand White (NZW) females 19 h after mating[20]. For microinjection the ova were placed in the well of a depression slide containing ~100 μl modified BMOC culture medium[21] with the $NaHCO_3$ replaced by 25 mM HEPES[22] and covered by silicone oil. Microinjection of embryos (1,857 one-cell and 50 two-cell) was performed as described for mouse ova[10,14]. Following injection, the ova were washed in fresh modified BMOC and surgically transferred to the oviducts of synchronized pseudopregnant rabbits[23].

† Rambouillet ewes were superovulated after exhibiting at least one prior oestrus period. On about day 10 of the oestrous cycle, progestagen-impregnated vaginal sponges (6α-methyl-17α-acetoxy progesterone, 60 mg; from Dr J. Lauderdale, Upjohn) were inserted and left for 12 days. Gonadotropin treatment (porcine follicle stimulating hormone, Burns) began three days before sponge removal and was continued twice daily (2.5 mg per injection, intramuscular) until the day following sponge removal[24]. At the onset of oestrus, ewes were either hand mated to fertile rams or inseminated *in utero* with 0.2 ml per horn of washed ram semen; 72 h after sponge removal, one-cell fertilized ova and cleaved ova were surgically collected from the reproductive tracts of anaesthetized ewes by flushing 6 ml Ham's F-10 medium containing 10% heat-inactivated fetal calf serum (FCS) from the utero-tubal junction through the cannulated infundibular end of each oviduct. The flushings were collected in sterile Petri dishes, and ova were removed under a dissecting scope. Ova were transferred to fresh Ham's F-10 containing 10% FCS and transported (~2.5 h) to Philadelphia in temperature-controlled containers. Microinjection of embryos (641 one-cell, 375 two-cell and 16 four-cell) was performed as previously described[10,14]. After embryos were injected, they were washed and transported to Beltsville. Embryos were aspirated into a glass micropipet tip with 10 μl Ham's F-10 and expelled 1-3 cm into the fimbriated end of the oviduct in synchronized recipient ewes. To assess the effects of transport and microinjection of DNA on egg development, a number of recipients bearing control and injected eggs were flushed 8 days following transfer. In recipients in which eggs were recovered, 26% of transported, uninjected and 10% of injected sheep eggs developed to blastocysts. Because of the high mortality of transported, injected eggs and the concern of multiple births, 5 or 6 embryos were transferred per recipient.

‡ Mature gilts were superovulated and bred as previously described[15]. At 18 to 27 h after the expected time of ovulation, gilts were anaesthetized and one cell fertilized the oviduct with 20 ml modified BMOC[21]. Ova were transferred to fresh BMOC and transported to Philadelphia. Microinjection of embryos (316 one-cell, and 1,719 two-cell) was performed as previously described[10,14]. The obscured pronuclei or nuclei or one- and two-cell pig ova were visible after centrifugation for 3 min at 15,000 *g*. Centrifugation of pig ova at this force and length of time has no detectable effect on development[15]. After embryos were injected, they were transported to Beltsville and transferred to the oviducts of recipient gilts as previously described[15]. To assess the effects of transport and microinjection of DNA on egg development, recipients bearing control and injected eggs were flushed 5 days following transfer. Approximately 52% of transported, uninjected and 23% of injected pig eggs developed to blastocysts. The pregnancy rate in recipients bearing only injected eggs was 50% while in recipients bearing both injected and control eggs 58% farrowed.

Table 2 Characteristics of transgenic rabbits, sheep and pigs

Species	Animal and sex	Gene copy (no. per cell)	*hGH* mRNA (molecules cell^{-1})	Immunoassayable hGH (ng ml^{-1})	Species	Animal and sex	Gene copy (no. per cell)	*hGH* mRNA (molecules cell^{-1})	Immunoassayable hGH (ng ml^{-1})
Rabbit	59-3*	20	0		Pig	100-3*	4	0	ND
	64-2♀	18	0			163-4*	140	0	ND
	68-3†	28	39	ND		3-2♂	330	26	Neg.
	68-4♀	24	0			3-6♂	490	53	17
	122-9♀	11	0			7-3♀	90	12	80
	131-8†	88	0			10-4♀	23	0	Neg.
	157-1♀	3	0			11-2♂	1	0	40
	163-3♂	3	15	250		16-3♀	3	0	Neg.
	167-5♀	10	0			16-8♀	10	18	53
	179-1♀	16	0			16-9♂	1	5	65
	179-2♂	36	0			17-4♀	3	0	Neg.
	179-5♀	6	0			18-3♀	3	6	60
	200-3*	8	920	ND		20-2♂	2	4	40
	221-1*	40	140	ND		20-8♂	110	1	2
	223-4*	5	0			21-4♂	1	2	Neg.
	223-5*	40	0			21-5♀	1	0	Neg.
						22-1♀	50	24	56
Sheep	693-1♀	1	ND	ND		23-8♀	7	41	730
						25-2♀	17	0	108
						25-4♀	2	0	Neg

A 2.6-kb linear fragment of the fusion gene *MT-hGH*[13] containing the mouse MT-I regulator/promoter fused to hGH[19] was injected into fertilized one-cell and two-cell rabbit, sheep and pig eggs as described for mouse ova[10,14]. The male or female pronuclei of one-cell ova and both nuclei of two-cell ova were microinjected with a 3 ng μl^{-1} solution of DNA in Tris-EDTA buffer[14]. The ova were transferred into the oviducts of recipients at the same stage post oestrus as the donors (see Table 1). Animals without identified gender were either killed as fetuses(*) or were stillborn(†). The number of foreign fusion genes per cell was estimated by extracting total nucleic acids from a piece of fetal liver, neonatal ear or tail samples and performing quantitative dot hybridization with a 1.0-kb *Pvu*II probe spanning most of the *hGH* structural gene[19] (see Fig. 2). *MT-hGH* mRNA was measured by solution hybridization with a ^{32}P-labelled oligonucleotide (21-mer)[25]. For rabbits, either a partial hepatectomy was performed or fetal liver was used. For pigs, the *MT-hGH* mRNA content of ear or tail samples was quantitated. The concentration of *hGH* was measured in pig plasma obtained shortly after birth and serum from a 9-month-old rabbit. Samples were assayed in duplicate at 2.5 and 10 μl by radioimmunoassay using a hGH kit provided by Dr Raiti (National Hormone and Pituitary Program). The assay did not cross-react with porcine GH but required extra normal rabbit serum and anti-rabbit gammaglobulin to quantify hGH in rabbit samples. Pigs with hGH values less than 2 ng ml^{-1} at birth were designated negative for hGH. At about one month of age, these pigs were also negative for hGH. ND, not determined; Neg, negative for hGH.

The key element in our success was the ability to visualize pronuclei and nuclei. Microinjection of the *MT-hGH* gene into the cytoplasm of 485 pig ova failed to produce DNA integration in 42 fetuses. Separate techniques for sheep and pigs were necessary because the opacity of the eggs differed. Although both contain dense cytoplasm, centrifugation did not help visualize pronuclei of sheep eggs and IC microscopy did not allow nuclear localization in pig ova. Preliminary work indicates

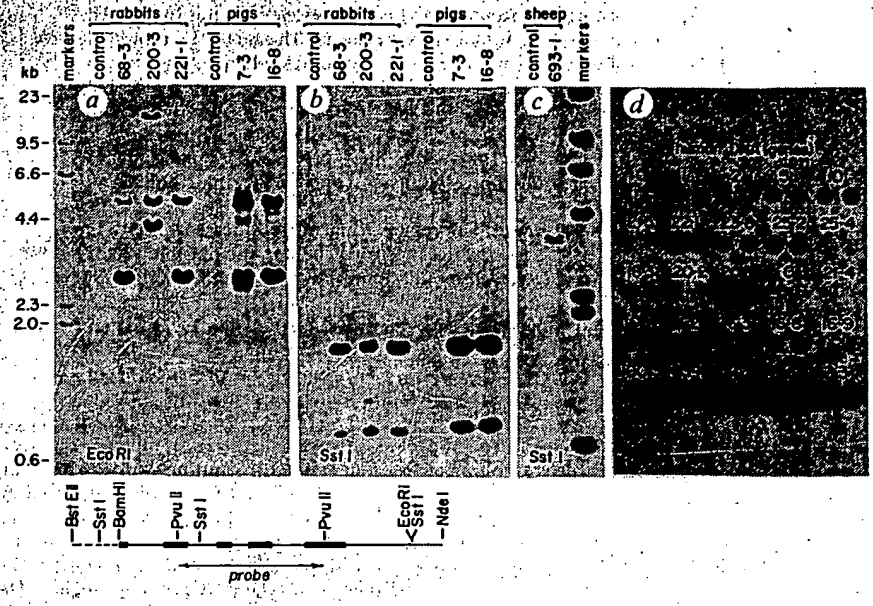

Fig. 2 Analysis of *MT-hGH* DNA introduced into rabbits, pigs and sheep. The diagram at the bottom shows the 2.6-kb *Bst*EII/*Eco*RI DNA fragment isolated from *MT-hGH* gene plasmid 111 that was microinjected[13]; the mouse MT-I promoter region is dashed, the *hGH* gene is solid, with the exons indicated as boxes, and the residual pBR322 sequences are dotted. The internal *Pvu*II fragment was isolated, nick-translated and used as a probe for quantitation of genes and Southern blots. Panels *a*, *b* and *c*: DNA (5 μg for controls and transgenic animals 200-3, 16-8 and 693-1; 1 μg mixed with 4 μg of control DNA for 68-3, 221-1 and 7-3) were digested with the indicated restriction enzymes (10 units, 6 h), electrophoresed on a 1% agarose gel, transferred to nitrocellulose and hybridized with the nick-translated probe, washed and autoradiographed as described previously[18]. *a* and *b*, Exposure was 4.5 h; *c*, 24 h. For quantitation of gene copy number, 5 μg of DNA was spotted in duplicate onto nitrocellulose along with standards of 0, 0.5, 1, 2 and 5 μg of human DNA mixed with control DNA to make a total of 5 μg. *d* Shows the visualization *MT-hGH* gene copy number in transgenic pigs. After exposure, the spots were cut out and the radioactivity determined in a scintillation counter. Gene copy numbers were calculated from the standards assuming that the genome size of pigs and humans are comparable and that diploid human cells contain 10 genes homologous to the *Pvu*II fragment used as probe[19]. The results are shown in Table 2.

that these two techniques can be used for ova of other species; for example, IC microscopy allowed visualization of pronuclei in goat ova (unpublished observations) and centrifugation allowed localization of pronuclei in cow ova[15]. Although improvements in integration efficiency should be possible, the techniques have immediate application for both scientific and practical purposes.

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Expression of active human clotting factor IX from recombinant DNA clones in mammalian cells

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Haemophilia B, or Christmas disease, is an inherited X-chromosome-linked bleeding disorder caused by a defect in clotting factor IX and occurs in about 1 in 30,000 males in the United Kingdom[1]. Injection of factor IX concentrate obtained from blood donors allows most patients to be successfully managed. However, because of impurities in the factor IX concentrate presently in use, this treatment involves some risk of infection by blood-borne viruses such as non-A, non-B hepatitis and the virus causing acquired immune deficiency syndrome (AIDS)[2]. Because of the recent concern about the increasing incidence of AIDS amongst haemophiliacs, a factor IX preparation derived from a source other than blood is desirable. Here, we report that after introduction of human factor IX DNA clones[3] into a rat hepatoma cell line using recombinant DNA methods, we were able to isolate small amounts of biologically active human factor IX.

Factor IX is a plasma glycoprotein which has an essential role in the middle phase of the intrinsic clotting pathway[4] where, in an activated form, IXa, it interacts with factor VIII, phospholipid and calcium ions to form a complex that converts factor X to Xa. Factor IX is synthesized in liver hepatocytes where it undergoes three distinct types of post-translational modification before secretion into the bloodstream as a 415-amino-acid-long, highly modified protein. These modifications are the vitamin K-dependent γ-carboxylation of 12 glutamic acid residues[5], the addition of several carbohydrate residues[6] and the β-hydroxylation of a single aspartic acid residue[7]. The first two modifications are known to be required for activity of factor IX[5,6]. Because of the complex and specialized nature of these modifications, it seemed probable that the expression of active factor IX, derived from factor IX DNA clones, would be most likely to succeed in a hepatic cell or a transformed cell line derived from a hepatocyte. None of the standard mammalian hepatoma cell

families (solid line) and acute families (dotted line). The peak multipoint lod score for chronic SMA is 9.03, and the peak lod score for acute SMA is 2.02. Pairwise lod scores for chronic and acute SMA families versus four markers located in the middle of the linkage region are shown in Table 1. The maximum two-point lod score for chronic families is 8.43 at a recombination fraction of 2% with marker D5S6, and 1.71 for acute families at a recombination fraction of 2% with marker D5S78.

Application of the HOMOG program[13] to the multipoint lod scores of the families with chronic SMA gave no evidence for heterogeneity among these families. Although the power of homogeneity tests can be lower in recessive families than in larger families with dominant diseases, the absence of evidence for heterogeneity led us to adopt the most parsimonious solution of assuming homogeneity. The confidence interval for the location of the gene for chronic SMA is 11 centimorgans (cM) wide and spans a region 2 cM proximal of locus D5S6 to a point 4 cM proximal of locus D5S78 (note arrows in Fig. 1). For families with acute SMA, the maximum lod score of 2.02 indicates that a gene responsible for this disease maps to the same general area. The best estimate for the location of the acute SMA locus is 15 cM distal to the estimated position of the locus for chronic SMA.

Our data indicate that clinically heterogeneous forms of chronic childhood SMA (type II or intermediate form and type III or Kugelberg-Welander or mild form) map to a single locus on chromosome 5q. The chronic forms of childhood-onset SMA, therefore, are likely to occur as the result of allelic heterogeneity, similar to the case for Duchenne- and Becker-type dystrophies[15]. It is interesting that our data indicate that acute childhood SMA (type I or Werdnig-Hoffmann or infantile SMA or severe SMA) map to the same, or a closely linked, locus on 5q. Other informative acute families must be analysed to confirm the linkage of this form of SMA and to evaluate the associated map location relative to that of chronic SMA. Also, other chronic families must be analysed to further assess the possible occurrence of nonallelic heterogeneity. It will be interesting to determine whether adult-onset and dominantly inherited cases of SMA similarly map to chromosome 5q. The gene encoding hexosaminidase B maps between markers D5S39 and D5S78 (refs 16, 17). Deficiencies in both the α- and β-subunit of this enzyme have been associated with chronic cases of SMA[18,19]. We are investigating whether this gene is a candidate for an SMA mutation. □

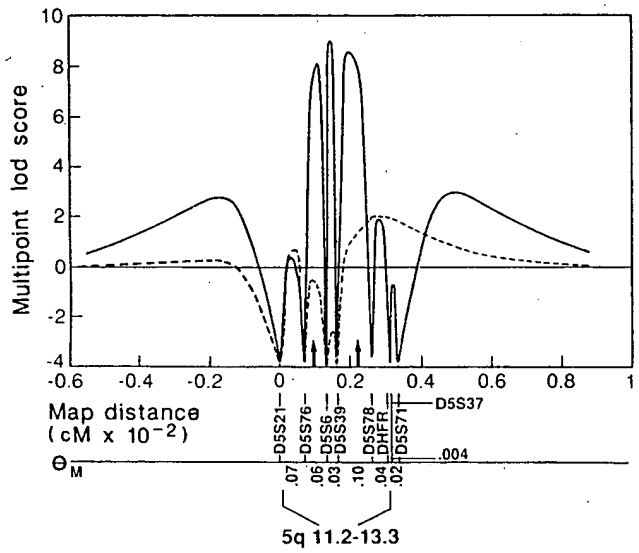

FIG. 1 Multipoint linkage analysis of the SMA disease locus with eight DNA markers spanning ~30 cM, including 5q11.2-5q13.3 (refs 16, 21). Analysis of seven chronic families (solid line) and six acute families (dotted line). Three chronic families each consists of four affected children and 8-12 unaffected sibs. Four chronic families each have three affected children and 0-4 unaffected sibs. The acute families, collected over a 3-year period, include one family with three affected children (trizygotic triplets), four families with two affected children, and one family with one affected and two unaffected sibs. Recombination fractions (θ_M) between DNA markers were calculated from published map distances[16]. Marker loci D5S6, D5S39, D5S78 and DHFR map to 5q11.2-13.3 (ref. 21). For the female-to-male distance ratio we used the published value of 1.6 as being appropriate for this area of the genome[22]. Multipoint lod scores were obtained by five-point analysis in all families, except one for which, for reasons of computational efficiency, three-point lod scores had to be calculated. The computer program used was LINKMAP of the LINKAGE package[20]. The confidence interval for chronic families (defined as points on the map with lod scores $\geq Z_{max}^{-1}$ where Z_{max} is the value of $Z(\theta)$ at the maximum likelihood estimate of θ) spans an 11-cM region marked by arrows at map positions 0.11 and 0.22.

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Fulminant hypertension in transgenic rats harbouring the mouse *Ren-2* gene

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PRIMARY hypertension is a polygenic condition in which blood pressure is enigmatically elevated; it remains a leading cause of cardiovascular disease and death due to cerebral haemorrhage, cardiac failure and kidney disease. The genes for several of the proteins involved in blood pressure homeostasis have been cloned and characterized[1-8], including those of the renin–angiotensin system, which plays a central part in blood pressure control[9-10]. Here we describe the introduction of the mouse *Ren-2* renin gene[3,11-13] into the genome of the rat and demonstrate that expression of this gene causes severe hypertension. These transgenic animals represent a model for hypertension in which the genetic basis for the disease is known. Further, as the transgenic animals do not overexpress active renin in the kidney and have low levels of active renin in their plasma, they also provide a new model for low-renin hypertension.

We chose the mouse *Ren-2* renin gene for introduction into the rat germline because it had already been characterized in transgenic mice and because we expected it to be highly

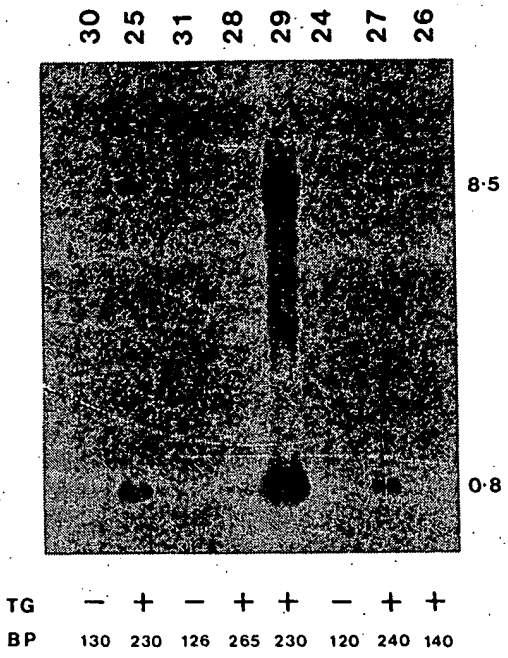


FIG. 1 Southern blot identifying animals carrying the DBA/2 *Ren-2* gene. The identification numbers of potential founder animals are shown above the corresponding lane and the positions of the *Ren-2*-specific 8.5-kb and 0.8-kb restriction fragments are indicated to the right. Transgenic (TG) positive and negative animals are indicated by symbols under the corresponding lane, together with the systolic blood pressure (BP, in mm Hg) of each animal at the age of 10 weeks.
METHODS. DNA preparation: DNA was prepared from tail biopsies and digested with *Pvu*II. After electrophoresis on a 0.8% agarose gel, samples were Southern-blotted and hydridized with a ^{32}P-labelled dCTP 300-bp probe derived from the renin complementary DNA clone pDD1D2[17] and labelled by random priming[18]. Preparation of transgenic animals: DNA was prepared for microinjection by digestion of the cosmid clone cosDBA-1 (ref. 17) with *Xho*I, and subsequent isolation of the 24-kb *Xho*I fragment containing the *Ren-2* gene on a 10–20% sucrose gradient in 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 200 mM sodium acetate. Fractions containing the required fragment were pooled and recovered by ethanol-precipitation before being centrifuged on a CsCl gradient[19]. DNA was diluted to a final concentration of 1 μg ml^{-1} in injection buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA), and stored in aliquots at −20 °C before use. Fertilized eggs were derived from a cross between Sprague-Dawley female and WKY male rats after superovulation of immature females (at 4 weeks old) according to the procedure of Armstrong *et al.*[20]. Eggs were cultured, microinjected, and re-implanted as described for the mouse[19]. Rats were all bred in our own facilities.

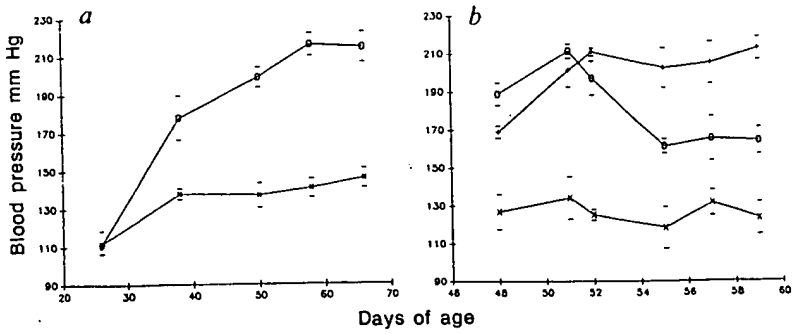
expressed in certain tissues[14]; also, injection of purified mouse submandibular gland (SMG) renin (encoded by *Ren-2*) into rats leads to a significant and sustained increase in blood pressure[15]. Fertilized rat eggs were microinjected with a linear DNA fragment containing the entire DBA/2J *Ren-2* gene, including 5.3 and 9.5 kilobases (kb) of 5′ and 3′ flanking sequence, respectively[14]. From 37 eggs implanted, there were eight progeny, of which five carried the transgene (Fig. 1). Four of the founders were bred successfully and three of them (TGRmRen2, numbers 25, 26 and 27) transmitted the transgene to their progeny. At ten weeks of age and before breeding, the blood pressure of the founder animals was measured. For four of the transgenic animals it was in the range 230–265 mm Hg, but was 120–130 mm Hg in the transgene-negative litter-mates (Fig. 1). Breeding of TGRmRen2 female 26, who was not hypertensive, revealed her to be mosaic for a transgene insertion site, the inheritance of which segregated with hypertension in the blood pressure range indicated (data not shown). The phenotype is therefore independent of the transgene insertion site and is not due to a fortuitous mutation associated with the integration event.

Analysis of the transgenic line established from TGRmRen2 male 27 revealed that, without exception, progeny inheriting the transgene also had the hypertensive phenotype. Both male and female animals of this line developed hypertension rapidly, beginning at four weeks of age and reaching a maximum by nine weeks (Fig. 2*a*). Pharmacological intervention to reduce blood pressure took the form of treating the animals with 10 mg kg^{-1} per day of the converting enzyme inhibitor, captopril; this inhibits the conversion of angiotensin I to angiotensin II. This low dose, given daily in the drinking water, was sufficient to reduce the blood pressure of the hypertensive transgenic rats reproducibly by 40–60 mm Hg (Fig. 2*b*), indicating that the hypertension is largely dependent on the conversion of angiotensin I to angiotensin II.

Northern blot analysis showed that the concentration of renin transcripts was high in the adrenal glands of the transgenic animals (Fig. 3*a*). In addition, renin transcripts were detectable in testis, coagulation gland, thymus and small intestine in transgene-positive animals, but not in control transgene-negative littermates (data not shown). These additional sites represent tissues in which renin is naturally expressed in the mouse. Renin messenger RNA was not observed in the SMG, a result that could reflect the absence of essential *trans*-acting factors in this tissue as the endogenous rat renin gene is not expressed in the SMG (ref. 16). An RNase protection assay using a *Ren-2*-specific probe confirmed that the renin transcripts in the adrenal gland were exclusively of *Ren-2* origin and that *Ren-2* transcripts were present in the kidneys of transgene-positive animals (Fig. 3*b*).

No evidence was found for altered plasma angiotensinogen levels, but plasma renin activity and angiotensin I were significantly lower in transgenic animals than in the controls (Fig. 4*b–e*). The amount of angiotensin II was also less than in the

FIG. 2 *a*, Development of blood pressure with age. Each point represents the mean of 7 (transgenic, circles) or 5 (control, crosses) animals and standard errors are indicated above and below each data point. *b*, Effect of converting enzyme inhibitor (CEI) on blood pressure. Each point represents the mean of 3 animals and standard errors are indicated above and below each data point. +, TGRmRen2 L27 rats having no treatment; ○, TGRmRen2 L27 rats receiving CEI; ×, control rats receiving CEI.
METHODS. Blood pressure was determined by tail plethysmography under light ether anaesthesia as described[21]. Animals under converting enzyme inhibitor treatment were given captopril (10 mg kg^{-1} per day) in their drinking water. Captopril treatment started at day 51.



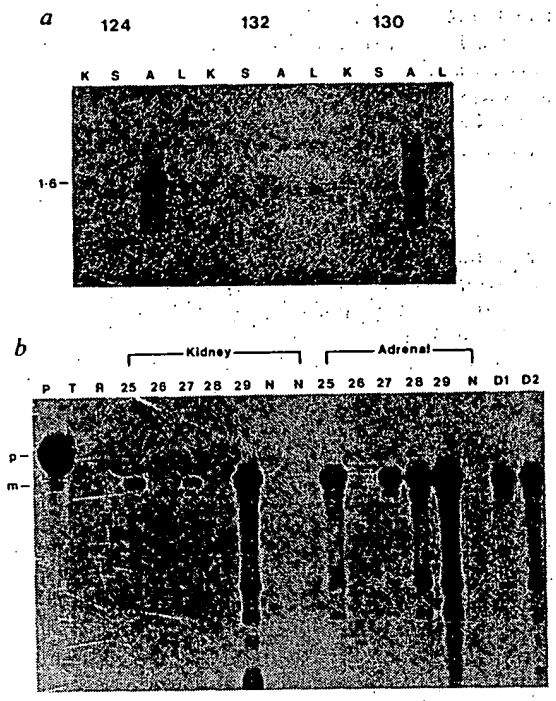


FIG. 3 Northern blot and RNase protection assay. *a*, Northern blot of RNA isolated from the kidney (K); SMG (S); adrenal gland (A); and liver (L) of transgene-positive (124 and 130) and transgene-negative (132) male rats. The size of the hybridizing RNA is indicated in kb. With the exception of the adrenal gland (5 μg), 40 μg total RNA was used for each sample. *b*, RNase protection of kidney and adrenal gland RNA from transgenic rats (numbers 25–29) and control littermates (N). The following controls are included: P, undigested probe; T, transfer RNA (9 μg); R, rat kidney RNA (20 μg); D1 and D2, mouse (DBA/2J) kidney RNA (20 μg and 40 μg, respectively). The positions of the undigested 244-nucleotide probe (p) and the 224-nucleotide mouse-specific protected fragment (m) are indicated.
METHODS. Preparation of RNA: Total RNA was isolated from mature rats as previously described[3] or by homogenization in guanidine isothiocyanate.[22] Northern blot analysis: Northern blots were prepared and hybridized as previously described[23] with a ^{32}P-labelled renin cDNA probe (pDD1D2) by random priming[18] and washed with 0.1 × SSC, 0.1% SDS at 65 °C. RNase protection assay: ^{32}P-labelled RNA transcripts were prepared by transcription of a 244-nucleotide antisense RNA from the plasmid pSLM (ref. 15) using SP6 RNA polymerase. This transcript comprised 224 nucleotides of *Ren-2* antisense RNA and 20 nucleotides of vector-encoded sequence. Samples were dissolved in 30 μl 80% formamide, containing 40 mM PIPES, 400 mM NaCl, 1 mM EDTA and 200,000 c.p.m. of the gel-purified transcript, denatured at 100 °C for 1 min and incubated at 45 °C for 20 h. RNase digestion was performed in 300 μl buffer containing 40 μg ml^{-1} RNase A (Sigma) and 2 μg ml^{-1} RNase T1 (Calbiochem) for 45 min at 37 °C. After digestion with proteinase K, samples were electrophoresed on denaturing 5% polyacrylamide gels.

controls but the difference was not statistically significant. Determination of prorenin showed it to be raised in the plasma of transgenic animals (Fig. 4*a*), but the functional significance of this finding is unclear. Adrenal glands of the transgenic animals contained significantly increased renin concentrations (Fig. 4*f*). No evidence was found for the storage of renin in this tissue, so the large difference between renin mRNA levels and enzyme activity may reflect a constitutive secretion of *Ren-2*-derived renin from the adrenal glands. By contrast, kidney tissue from transgenic animals contained only 20–25% of the renin activity of the controls, which is consistent with immunocytochemical and ultrastructural data showing a reduction in renin storage granules in the juxtaglomerular apparatus (S. Bachmann *et al.*, manuscript in preparation) and suggests that renin expression is subject to translational or post-translational control. Preliminary studies on isolated kidney show that renin secretion is reduced and that there are no other abnormalities of renal function (K. Munter, personal communication).

Although we have defined a genetic basis for this transgenic hypertensive rat model, the mechanism responsible for elevating blood pressure remains to be established. The hypertension is clearly not due to overexpression of renin in the kidney, and the suppression of active renin in the kidney and in the plasma is probably a result of an already elevated blood pressure in young animals, pressure-mediated renin suppression being a well known phenomenon. The increased plasma prorenin probably originates, at least in part, from the adrenal gland, but the ovary, vascular tissue and other sources of prorenin should also be considered. Any role of prorenin in hypertension still awaits investigation, but in this respect it is interesting that prorenin is raised and still persists after nephrectomy in hypertensive patients, confirming that its origin is extra-renal. At this stage, the most likely explanation for the high blood pressure in TGRmRen2 rats is a stimulated renin–angiotensin system in the adrenal gland, with the consequent overproduction of steroid hormones. This is in keeping with our preliminary data on

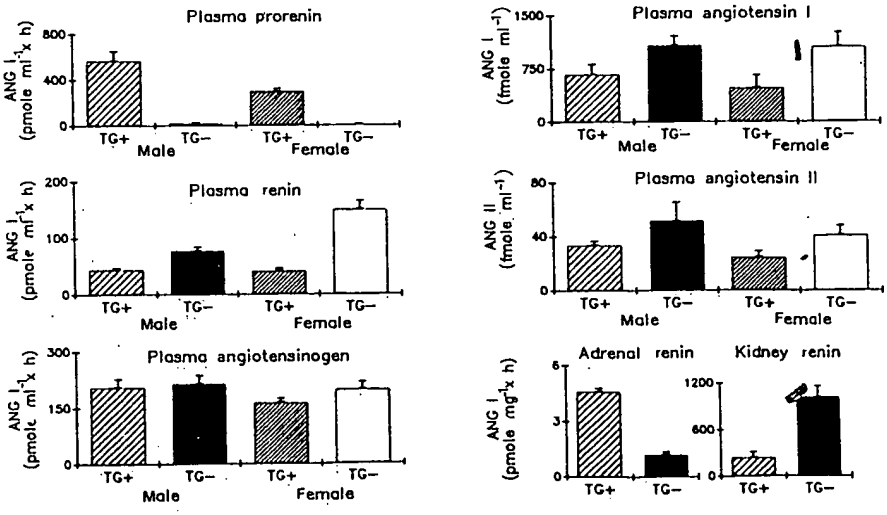


FIG. 4 Determination of plasma and tissue renin-angiotensin system components. Values represent the mean and standard error of 7 animals for each determination, with the exception of the kidney and adrenal gland renin values (3 animals). Statistical analysis by ANOVA showed the following significance values: prorenin, $P < 0.05$ between the transgenic animals and the corresponding controls; renin, $P < 0.005$ between the transgenic animals and the corresponding controls; angiotensin I, $P < 0.05$ between the transgenic animals and the corresponding controls; tissue renin, $P < 0.01$ for the adrenal gland and $P < 0.005$ for the kidney.
METHODS. Concentrations of angiotensinogen, angiotensin I, angiotensin II and renin were determined as described[24–25]. Prorenin levels were calculated by subtraction of renin activity from total plasma renin activity determined after trypsin activation[26].

elevated urinary aldosterone excretion in male TGRmRen2 rats (15.4 ± 2.26 ng per 24 h) compared with controls (8.97 ± 1.06 ng per 24 h). These animals will enable us to study normal or low plasma renin hypertension and have shown us that renin can participate in the genesis of hypertension in a more subtle way than previously supposed. The construction of transgenic rats will therefore provide new opportunities for research into cardiovascular mechanisms. □

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Imprinting of acetylcholine receptor messenger RNA accumulation in mammalian neuromuscular synapses

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In mammalian muscle, the subunit composition of the nicotinic acetylcholine receptor (AChR) and the distribution of AChRs along the fibre are developmentally regulated. In fetal muscle, AChRs are distributed over the entire fibre length whereas in adult fibres they are concentrated at the end-plate[1]. We have used *in situ* hybridization techniques to measure the development of the synaptic localization of the messenger RNAs (mRNAs) encoding the α-subunit and the ε-subunit of the rat muscle AChR. The α-subunit is present in both fetal and adult muscle, whereas the ε-subunit appears postnatally and specifies the mature AChR subtype[2–4]. The synaptic localization of α-subunit mRNA in adult fibres may arise from the selective down-regulation of constitutively expressed mRNA from extrasynaptic fibre segments. In contrast, ε-subunit mRNA appears locally at the site of neuromuscular contact and its accumulation at the end-plate is not dependent on the continued presence of the nerve terminal very early during synapse formation. This suggests that ε-subunit mRNA expression is induced locally via a signal which is restricted to the end-plate region and is dependent on the presence of the nerve only during a short period of early neuromuscular contact. Evidently, several mechanisms operate to confine AChR mRNAs to the adult end-plate region, and the levels of α-subunit and ε-subunit mRNAs depend on these mechanisms to differing degrees.

Hybridization of longitudinal sections of adult rat soleus muscle with ε- and α-subunit-specific antisense complementary RNA (cRNA) probes revealed strong hybridization signals at sites that had been previously identified as end-plates by staining for acetylcholinesterase (AChE). Figure 1*a* shows the end-plate region of a muscle stained for AChE. Subsequent hybridization with the ε-subunit-specific antisense probe showed a strong signal at the site where the AChE stain had been (Fig. 1*b*). After a brief exposure, groups of grains could be resolved above individual synaptic nuclei (Fig. 1*c*); no hybridization was observed outside end-plate regions. When sections were incubated with ε-subunit-specific sense probes, no hybridization could be detected (data not shown). These observations suggest that autoradiographic grain clusters reflect locally increased ε-subunit mRNA levels below the end-plate membranes. Similar results were obtained after hybridization with α-subunit-specific antisense (Fig. 1*d*, *e*) and sense probes and confirm the synaptic localization of α-subunit mRNA in rat muscle, as observed previously using northern blot analysis[5]. In some fibres, a small signal was observed above nuclei in the perijunctional region of the muscle fibres (Fig. 1*e*).

Previous northern blot analysis of AChR-specific mRNAs in neonatal rat muscle indicated that ε-subunit mRNA is barely detectable at birth but that levels increase rapidly during the first 2 weeks of postnatal development[4]. To determine whether this increase in ε-subunit mRNA is restricted to the end-plate region and therefore would be induced focally by the nerve, or whether the increase is more general, involving the entire fibre, we hybridized triceps muscle from rats of different postnatal ages with an ε-subunit mRNA-specific cRNA probe. Figure 2*a* shows the localization of AChE and autoradiographs of longitudinally sectioned muscle (*b–d*). At postnatal day 1, no hybridization signal could be detected either synaptically or extrasynaptically (Fig. 2*b*). In dark-field microscopy, some of the synaptic sites revealed a weak accumulation of grains (data not shown). However, on postnatal days 5, 9 (data not shown) and 12, an increasingly stronger signal was seen (Fig. 2*c*) that always coincided with the AChE-stained synaptic sites. Thus, the postnatal appearance of ε-subunit mRNA is restricted to the end-plate region from the earliest stages of synapse development and therefore must be induced by the nerve-muscle contact. As in adult muscle, hybridization signals in postnatal day-12 muscles were clearly associated with individual nuclei, as shown in Fig. 3. However, given the high density of nuclei from various cell types, unequivocal attribution to subneural nuclei was not always possible.

In contrast, total α-subunit mRNA remained at a plateau level during the first 12 postnatal days[4]. During this period, the α-subunit mRNA was detected throughout the fibre, in both the synaptic and extrasynaptic fibre segments (Fig. 2*f*, *g*). Although there were more grains at the synaptic sites, they were more widely distributed than those obtained upon hybridization with the ε-subunit mRNA specific probe. Moreover, the hybridization signal was also observed outside the myofibre bundles above unfused cells.

The level of total muscle ε-subunit mRNA increases almost normally in neonatal muscle denervated shortly after birth[4], indicating that only the brief, prenatal nerve-muscle contact is necessary to induce ε-subunit mRNA synthesis. We have investigated whether the ε-subunit mRNA still appears focally at the

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Strain-dependent differences in the efficiency of transgenic mouse production

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Abstract

Transgenic mouse production via pronuclear microinjection is a complex process consisting of a number of sequential steps. Many different factors contribute to the effectiveness of each step and thus influence the overall efficiency of transgenic mouse production. The response of egg donor females to superovulation, the fertilization rate, egg survival after injection, ability of manipulated embryos to implant and develop to term, and concentration and purity of the injected DNA all contribute to transgenic production efficiency. We evaluated and compared the efficiency of transgenic mouse production using four different egg donor mouse strains: B6D2/F1 hybrids, Swiss Webster (SW) outbred, and inbred FVB/N and C57BL/6. The data included experiments involving ~350 DNA transgene constructs performed by a high capacity core transgenic mouse facility. Significant influences of particular genetic backgrounds on the efficiency of different steps of the production process were found. Except for egg production, FVB/N mice consistently produced the highest efficiency of transgenic mouse production at each step of the process. B6D2/F2 hybrid eggs are also quite efficient, but lyze more frequently than FVB/N eggs after DNA microinjection. SW eggs on the other hand block at the 1-cell stage more often than eggs from the other strains. Finally, using C57BL/6 eggs the main limiting factor is that the fetuses derived from injected eggs do not develop to term as often as the other strains. Based on our studies, the procedure for transgenic mouse production can be modified for each egg donor strain in order to overcome any deficiencies, and thus to increase the overall efficiency of transgenic mouse production.

Introduction

Analysis of transgenic mice has become a key approach for studying the function of genes in the context of the whole organism, as well as for modeling human diseases. A centralized transgenic mouse facility provides an efficient and economical way to produce transgenic mice for a large number of researchers who are engaged in a wide variety of scientific disciplines, since expensive specialized equipment does not need to be purchased or the critical technical skills acquired. The present article addresses two subjects pertaining to the efficiency of transgenic mouse production: the effect of different genetic backgrounds of the egg donors on transgenic production, and what are the ideal measurements for evaluating transgenic mouse production.

The production of transgenic mice by injection of DNA into the pronucleus of a zygote became a firmly established technique during the 1980s, when

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optimized protocols for the procedure were published (Brinster et al., 1985; Hogan et al., 1986). The Induced Mutant Resources (IMR) database at Jackson Laboratories lists approximately 260 transgenic lines that are available for distribution, and hundreds of additional transgenic mouse lines are maintained in individual laboratories. In addition, many transgenic mice are not saved as lines after a study is completed. Consistent with this wide use of transgenic mice in biomedical research, a policy of the National Institutes of Health (US) is that the proportion of funds directed towards mouse models should rise to stimulate a substantial increase in the number of transgenic animals produced (Croy, 2000). In order to ensure that the transgenic mice are produced efficiently and in an economic manner, it is important to establish uniform criteria for evaluating the efficiency of transgenic production and to identify the key parameters that influence transgenic mouse production.

For many experiments involving the use of mice, the choice of genetic background is very important for the outcome (for example: Dandekar & Glass, 1987; Roudebush & Duralia, 1996; Scott & Whittingham, 1996; Sztein et al., 2000). To date, only a handful of papers have attempted to address factors that influence the efficiency of transgenic mouse generation and the choice of genetic backgrounds. Brinster and his colleges analyzed the efficiency of transgenic mouse production with regard to an optimized microinjection procedure, in terms of DNA concentration, size and form, the site of injection, and buffer composition (Brinster et al., 1985). The study also noted that generating transgenic mice with a hybrid line was eight times more efficient than with inbred C57BL/6 mice, and concluded that the overall efficiency of transgenic production can be influenced by the choice of mouse strains. Three other publications contain results of DNA microinjection using different strains, but less than one thousand eggs were manipulated in each case (Taketo et al., 1991; Canseco et al., 1994; Osman et al., 1997). With these small numbers there was considerable variation between studies. One more paper (Paris et al., 1995) reported efficiencies with FVB/N and two hybrid mouse lines that were ten fold lower than those shown by the other studies and attributed this to the transgenes used. Furthermore, in a technical guide for making mouse transgenics (Mann & McMahon, 1993) the authors provided results of manipulating eggs from their laboratory using only one donor strain (B6CBA/F1) and reported an unusually high efficiency of transgenic production, but did not point to the factor(s) that specifically promoted such a high result. Updated and more extensive analysis therefore is needed to determine the contribution of strain-dependent factors to the efficiency of transgenic mouse production.

In the present study we analyzed the results of injecting tens of thousands of eggs from four strains of mice. The data were collected over a four-year period to overcome the normal fluctuations seen in transgenic production due to subtle changes in laboratory conditions. Our studies enabled us to identify key characteristics of the four strains that significantly influence the efficiency of transgenic mouse production.

Materials and methods

DNA construct purification and quantification

Transgenic constructs were prepared in over 30 individual laboratories within NYU School of Medicine and were made according to our recommended protocol. DNA containing the expression cassette was separated from the vector and purified from an agarose gel slice by electroelution, followed by phenol/chloroform extraction and dialysis in TE (dialysis tubing from Invitrogene, previously Gibco BRL, #15961-022, Collodion bag from Sartorius Corp., #13200), or buffer exchange using Centriprep-30 concentrators (Millipore, #4306), followed by ethanol precipitation and washes in 70% ethanol. DNA was resuspended in microinjection buffer (MIB: 10 mM Tris pH 7.4, and 0.15 mM EDTA pH 8.0) at a concentration of at least 40 ng/μl. DNA was stored frozen at −20°C. The bacterial artificial chromosome (BAC) DNA purification method involved alkaline lysis, followed by double acetate precipitation of DNA and then purification of the BAC DNA on a CsCl gradient, followed by buffer exchange using Centriprep-30 concentrators. The samples were stored at 4°C in TE. Before injection, BAC DNA was diluted to ~0.3–2 ng/μl in MIB containing 30 μM spermine and 70 μM spermidine.

The transgenic facility staff analyzed the DNA by gel electrophoresis. For constructs smaller than 10 kb the DNA concentration, construct size and integrity (lack of smearing or additional bands) were evaluated by agarose gel electrophoresis. A series of sample dilutions were run on a 1% agarose gel with a DNA

ladder standard (High DNA Mass Ladder, Invitrogene, previously Gibco BRL, #10469-016) of a known amount of DNA for each band. For plasmid constructs larger than 10 kb, DNA concentration was measured using a fluorometer (Hoefer, DQ200) and an agarose gel was run to evaluate the integrity of the DNA. Only DNA constructs that were a single band on an agarose gel were used for injection. For BAC DNA, integrity was determined using pulsed field gel electrophoresis.

Generation of transgenic mice

DNA was injected into the male pronuclei of oocytes according to the procedure described in *Manipulating the Mouse Embryo* (Hogan et al., 1986). DNA was thawed and diluted to a concentration of 2–5 ng/μl on the day of injection. For one day of injection (defined as an experimental day) 15 females were superovulated and mated, embryos were dissected from plug positive females and all fertilized eggs were used for injections. The vast majority (90%) of experiments involved manipulation of at least 120 eggs (range: 90–250). The light cycle in the mouse rooms was 5 PM off and 3 AM on. Donor females were injected intraperitonealy with 5 IU pregnant mare's serum (PMSG, Calbiochem, #367222) at 3 PM and with 5 IU human chorionic gonadotropin (hCG, Pregnyl, Organon Inc., #0052-0315-10) 46 h later (1 PM) and immediately mated with appropriate stud males. The males were monitored for their breeding performance by keeping a record of plugging. Males that did not mate on several occasions (4–6) were removed from the facility. For C57BL/6 males, an additional test was performed which involved placing a female in the cage and checking whether the male failed to fertilize the female after two weeks (lack of implantation sites in the uterus). We observed that the frequency of mating is strongly influenced by two factors: how often males are used and the age of donor females. Once we optimized these two factors there was no difference in the plug rate between strains.

Microinjections were performed by four experienced technicians with an Eppendorf Transjector 5246. After injection of DNA into the pronucleus, embryos were cultured overnight (17–26 h) in M16 medium (Specialty Media, MR-016-D) at 37°C and 6–7% CO_2, unless otherwise noted in the results. Hormones, media and culturing conditions were routinely monitored for optimal quality by recording the number of embryos obtained on a daily basis and by periodic controls involving culture of non-manipulated zygotes to the blastocyst stage, followed in some cases by embryo transfer. The day after pronuclear injection the embryos that had divided to the 2-cell stage were implanted into Swiss Webster (SW) foster mothers. Approximately 15 embryos were transferred into each oviduct of each recipient. In a few cases ~35 embryos were transferred into one female. In the results presented, we included experiments in which one of the recipients from an experiment died. The number of embryos that lyzed after the injection procedure was recorded. Similarly, the number of embryos that underwent a 1-cell block was recorded. Progeny were genotyped by PCR and/or Southern blot analysis in individual investigator's laboratories. In ~15% of the experiments founder embryos were examined for lacZ expression by X-Gal staining and not genotyped, thus the actual number of transgenics was likely higher.

Mice

Outbred SW, hybrid B6D2/F1, and inbred FVB/N, C57BL/6 or 129S6/SvEv mice were purchased from Taconic Farms. F2 hybrid zygotes for manipulation were obtained by interbreeding B6D2/F1 mice.

Databases

Custom designed FileMaker Pro databases, kindly provided by Tom Clarke (NYU Kaplan Comprehensive Cancer Center), were used for weekly scheduling, record keeping, and summarizing results. The weekly scheduling database allowed technicians to sign up for superovulated females from each of the four strains, and naturally mated or pseudopregnant females. Signing up was organized by plug detection date. The database generated a weekly schedule with dates for hormone injections, animal mating and plug checking. To keep track of the results of microinjection experiments a separate microinjection datasheet was used for each construct. The datasheet included general data such as: laboratory submitting the request, the DNA construct name and size, individual laboratory and core facility evaluation of DNA sample concentration, and dilution used for microinjection. Each row of the table represented one experimental day. For each experimental day the number of plug positive females was recorded, as well as the number of fertilized eggs recovered, number of eggs injected, number of eggs that lyzed, or underwent 1-cell blocks, date of embryo transfer, number of eggs transferred, number of pseudopregnant females used for the transfer, number of females which got pregnant, number of pups

Table 1. Quantity and quality of embryos obtained from superovulated females by donor strains. Significance evaluated by the standard Z-test

Donor strain	Average no. of fertilized eggs/ experiment[a]	STD error of means	Egg survival = % total transferred[c] / total eggs injected	Observations
FVB/N	149.88[b]	3.27	80.26	Inconsistent response to hormones, large pronuclei, eggs advance quickly to 2-cell stage, frequent mosaic founders
B6D2/F1	167.88[b]	3.11	72.55	Consistent number of fertilized eggs with accessible pronuclei
SW	148.01[b]	2.38	61.38	Large fraction of poor quality embryos
C57BL/6	155.92	7.72	71.32	Asynchronous and slow development of eggs with small poorly visible pronuclei

[a] Fifteen females were superovulated and mated (rate of mating can be considered as part of breeding performance of the strain).
[b] Statistically significant difference between B6D2/F1 and SW or FVB/N (at 1% level).
[c] Subjective evaluation of suitability of embryos for transfer into recipients was applied in which all 2-cell stage embryos were used and some elongated 1-cell stage embryos were also transferred.

born, and number of transgenics reported by the individual laboratory. Any unusual observations were also recorded.

Statistical evaluation

Significance of the differences between results was evaluated by the standard Z-test based on the normal distribution.

Results

The data used for the studies presented is from all experiments conducted in a period of four years. Over this period, 346 DNA transgene constructs were injected into zygotes on 797 experimental days. Eggs with four different genetic backgrounds were used (B6D2/F2 hybrid, FVB/N or C57BL/6 inbred, and SW outbred) and 2268 transgenic mice were produced. The results of seemingly failed experiments (e.g., a large proportion of the injected eggs did not survive manipulation, a very low number of babies were born, or some of the recipients did not give birth) were included in the data to allow us to investigate the reasons for such unfavorable outcomes. The overall transgenic mouse production efficiency is expressed as a percentage of the number of transgenics obtained from the number of eggs injected.

Strain-dependent effects on donor egg production

A major factor influencing transgenic mouse production is the quality of donor eggs and the efficiency of producing them. Table 1 summarizes the results of egg production for four donor strains. Fifteen females were superovulated for each experimental day and the average daily number of fertilized eggs that were produced for each genetic background was calculated. Statistically significant differences were identified between B6D2/F1 and FVB/N or SW donor mice, with B6D2/F1 hybrid intercrosses producing a higher number of fertilized eggs per day than FVB/N or SW (~168 v.s. ~150 and ~148, respectively). C57BL/6 mice produced an intermediate number of donor eggs (~156).

Tolerance of eggs to DNA microinjection

Egg survival was evaluated only for experiments in which embryo transfers were performed on the day after injection. An egg survival percentage was calculated for each strain as the number of eggs that survived overnight culture and were suitable for transfer into recipients (in most cases the eggs had divided to the 2-cell stage), from the total number of fertilized eggs that were injected. Table 1 presents the results of injection of tens of thousands of eggs. FVB/N eggs seemed to have the highest egg survival (~80%), whereas SW eggs had the lowest survival rate (~60%) between the four strains.

To identify factors that lower egg survival, both loss of injected eggs due to lysis soon after injection and due to a block in cell division (1-cell block) were evaluated. We detected a significantly higher rate of lysis for B6D2/F2 eggs (~22%) compared to FVB/N and C57BL/6 eggs (~16 and ~18%, respectively). A

Table 2. Results of DNA microinjections by donor strain. Significance evaluated by the standard Z-test

Donor strain	No. of eggs injected	% Eggs lyzed/ injected	% 1-Cell blocks/ injected	% Born/ injected	% Born/ transferred[e]
FVB/N	28,608	15.73[a]	4.41	16.34[c]	20.28
B6D2/F1	30,369	22.10[a]	5.12	13.36[c]	18.56
SW	54,027	20.85	17.87[b]	11.67	19.43[d]
C57BL/6	7139	17.84[a]	10.86	9.43[c]	13.87[d]

[a] Significantly higher for B6D2 than for FVB/N or C57BL/6 (at 1% level).
[b] Significantly higher for SW than for other three strains (at 1% level).
[c] Significantly lower for C57BL/6 than for FVB/N or B6D2/F1 (at 1% level).
[d] Significantly lower for C57BL/6 than for SW (at 1% level).
[e] Subjective evaluation of suitability of eggs for transfer into recipients was applied. All 2-cell stage embryos and some elongated 1-cell stage embryos were transferred.

high percentage of SW egg lysis (~21%) was also observed. What was striking, however, was that the number of SW eggs that blocked at the 1-cell stage following DNA injection was much higher (at 1% level) compared to FVB/N, B6D2/F2 or C57BL/6 eggs (~18% v.s. ~4%, ~5% and ~11%, respectively; see Table 2).

All eggs that were at the 2-cell stage after overnight culture were transferred into SW foster mothers on the next morning. The percentage of pups born from the total number of injected eggs was significantly lower (at 1% level) for C57BL/6 (~9%) than for FVB/N (~16%) and B6D2 hybrids (~13%). There also was a significant difference in the percentage of pups born from the number of eggs transferred between C57BL/6 and SW. By using the number of embryos transferred rather than injected, the influence of egg loss due to lysis or 1-cell blocks is not included in the measurement. The frequency of pregnancy was not found to vary greatly between the four strains of eggs used (80–91%), based on visual inspection of recipients at mid-gestation, thus this cannot be the primary reason for the low number of mice born for C57BL/6 2-cell stage embryos. The pregnancy rate for injected C57BL/6 embryos (~85%) was only slightly lower than for FVB/N embryos (~91%). Furthermore, some of the female recipients of C57BL/6 manipulated eggs thought to be pregnant, but which failed to deliver, showed resorption sites when autopsies were performed soon after the due dates. Consistent with a loss of embryos during late development in utero, in a control experiment in which C57BL/6 embryos were transferred into recipients without DNA injection or overnight culture, only ~28% developed to term, although the majority of the mothers gave birth (~86%).

Evaluation of transgene DNA quality

We found that the quality of DNA prepared for microinjection can greatly influence the overall production efficiency. Over the four-year period of operation of the core facility some laboratories consistently provided DNA that was more 'sticky' or 'difficult' to inject compared to other laboratories. The ease with which a transgene could be injected was recorded in the microinjection datasheet. We observed six cases in which the first sample of a DNA construct caused a high number of 1-cell blocks after overnight culture and produced no transgenics, whereas an independently purified second sample of the same construct produced few 1-cell blocks and transgenic mice. In these cases the difficulties could be attributed to the quality of the DNA sample, rather than to a specific construct. Experiments with all DNA preparations are included in the data presented.

As a possible means to evaluate the influence of DNA purity on transgenic production we compared the results obtained from each of two laboratories that made a large number of transgenic constructs with the cumulative results of nearly 20 laboratories that made transgenic constructs less frequently (Table 3). To remove the variable of genetic background of the egg donor, results from one strain (SW) were compared. Group A contained results of 53 constructs from 18 different laboratories submitting constructs infrequently (1–8 constructs/laboratory), group B contained results from one laboratory for 37 injected constructs and group C contained results from yet another laboratory for 29 constructs injected. The overall transgenic production efficiencies (% transgenics of eggs injected) for the two more experienced groups were higher (at 1% level) than for the inexperienced

Table 3. Comparison of transgenic production efficiencies expressed as percentage of transgenics obtained from eggs injected for laboratories with different experience in making transgene DNA. Significance was evaluated by the standard *Z*-test

Strain	Group (no. of laboratories)	No. of constructs	% Eggs lyzed/ injected	% 1-Cell blocks/ injected	% Born/ injected	% Transgenics/ eggs injected
SW	A (18)	53	21.1[a]	20.5[b]	10.7[c]	1.5[d]
	B (1)	37	18.4[a]	15.3[b]	14.2[c]	2.1[d]
	C (1)	29	19.8	19.0	12.8	2.3[d]
FVB/N	D (19)	57	15.7	4.4	15.6	2.7[e]
	E (1)	19	15.2	4.6	19.2	4.5[e]

[a] Statistically significant difference between two groups. Results for group A that made transgenic constructs less frequently were higher than for experienced group B (at 5% level).
[b] Statistically significant difference between two groups. Results for group A were higher than for group B (at 1% level).
[c] Statistically significant difference between two groups. Results for group A were lower than for group B (at 1% level).
[d] Statistically significant difference in results for laboratories that made a large number of transgenic constructs (groups B and C) than for group A (at 1% level).
[e] Statistically significant difference between two groups. Results for experienced group E were higher than for group D that made transgenic constructs less frequently (at 5% level).

group (A – 1.5%; B – 2.1%; C – 2.3%). Similarly, we analyzed the overall transgenic production efficiency for laboratories using FVB/N egg donors. Group D contained results of 57 constructs from 19 different laboratories that produced transgenic constructs infrequently (1–8 constructs) and group E contained results from one laboratory for 19 constructs. The overall results of groups D and E were significantly different at 5% level (D – 2.7%; E – 4.5%). In an attempt to determine at what step of the procedure poor DNA impedes the outcome we statistically analyzed each step of transgenic production for both strains and found that for the SW background that the less experienced laboratories were less efficient in all steps than one of laboratories producing transgenic mice more frequently. This suggests that poor quality DNA may affect each step of production with the cumulative lowering of the overall transgenic production efficiency.

Influence of time of embryo transfer on transgenic production

Most of the published papers relating to procedures for transgenic mouse production (Hogan et al., 1986; Mann & McMahon, 1993; Pinkert, 1994; Hammes & Schedl, 2000) describe transferring the embryos to recipient mothers either soon after the DNA injection is performed, or on the next morning. Mann and McMahon (1993) reported similar efficiencies of transgenic production for both times of embryo transfer for hybrid eggs. The majority of DNA microinjection experiments in our data set involved transferring the embryos on the morning after the DNA injection. On some occasions, however, eggs were transferred on the same day. We compared the results of 28 experiments in which eggs were injected with the same construct and transferred on the same day or transferred on the next day (Table 4). For SW egg donors the percentage of pups born from the injected eggs was significantly higher (at 5% level) when the eggs were transferred on the same day as the DNA injection compared to on the next day (14.4% v.s. 9.7%). Surprisingly, the percentage of transgenic pups produced from the number of eggs injected was also significantly (10% level) higher when eggs were transferred on the day of microinjection rather than on the next day (2% v.s. 1.1%, respectively). This suggests that the uterus provides a more optimal environment than *in vitro* culture, specifically for transgenic eggs. For FVB/N and hybrids no statistically significant differences were observed when injected eggs were transferred to recipients on the same or next day.

Measurements of transgenic production efficiency

In Table 5 we show three different ways of evaluating the transgenic production efficiency in the

Table 4. Comparison of the efficiency of transgenic production using two times of embryo transfer. Significance is evaluated by the standard Z-test

Donor strain	Same day embryo transfer			Next day embryo transfer		
	No. of eggs injected	% Born/ injected	% TGs/ injected	No. of eggs injected	% Born/ injected	% TGs/ injected
FVB/N	1244	18.9	4.7	849	17.3	3.6
B6D2/F1	841	15.0	2.0	963	15.3	1.7
SW	1970	14.4[a]	2.0[b]	1877	9.7[a]	1.1[b]

[a] Significant difference of results between two times of transfer (at 5% level).
[b] Significant difference of results between two times of transfer (at 10% level).
Note lack of significant differences between two times for FVB/N and hybrid strain.
TGs: transgenics.

Table 5. Overall efficiency measurements of DNA microinjections by donor strains. Significance evaluated by the standard Z-test

Donor strain	No. of TGs/ experimental day	% TGs/ injected eggs	Maximum % TGs/ injected eggs obtained	% TGs/ born[b]
FVB/N	4.1	3.0[a]	17.63	17.4
B6D2/F1	3.3	2.1[a]	5.36	16.5
SW	2.1	1.7[a]	8.11	15.1
C57BL/6	1.8	1.2[a]	3.37	14.9

[a] All values significantly different (at 1% level).
[b] TGs: transgenics. Note lack of statistically significant differences.

four different genetic backgrounds: (i) the number of transgenic mice produced per experimental day; (ii) the percentage of transgenics produced from the number of eggs injected; and (iii) the percentage of transgenics produced from the number of animals born. For our facility, which has a user fee based on a defined injection day; it can benefit the customer to use a generic term 'the number of transgenics obtained per experimental day'. This number allows investigators to anticipate the cost of transgenic production depending on the number of transgenic animals required. The overall transgenic production efficiency measured as the percentage of transgenic animals produced from the number of eggs injected was found to be significantly different for all strains (at 1% level). The highest overall transgenic production efficiency (3.0%) was obtained with FVB/N eggs. Furthermore, the transgenic production rate was higher for B6D2/F2 hybrid eggs (2.1%) than for SW (1.7%) and C57BL/6 (1.2%) eggs. Of interest, there were no statistically significant differences in the percentages of transgenic mice produced from the number of mice born between the four strains. Thus, the main limitations to transgenic production come not from the injection procedure (once the procedure is optimized) or the frequency of DNA integration between different strains, but from embryo viability and resistance to the injection procedure and culture conditions. The efficiency of egg production is also a consideration when comparing FVB/N, SW and B6D2/F1 mice and this is reflected in the number of transgenics made per experimental day.

Discussion

There are many factors that influence the efficiency of transgenic mouse production and previous studies suggested that the genetic background of the egg donor could influence the overall efficiency of transgenic production (Brinster et al., 1985; Taketo et al., 1991; Mann & McMahon, 1993; Paris et al., 1995; Osman et al., 1997). In the present studies we have extended these findings considerably by analyzing a much larger data set and directly comparing four different mouse strains. Our studies allowed us to identify particular steps in the production of transgenic mice that

are significantly different between strains; response of egg donor females to superovulation, viability of eggs following injection, tolerance of injected eggs to overnight culture, and the ability of embryos to implant and develop to term.

Certain measurements of transgenic production efficiency can identify strain-dependent factors

To evaluate the efficiency of transgenic production in detail, several measurements should be considered (Tables 1, 2 and 5) to uncover the cause(s) of a lower than expected transgenic production efficiency. A commonly used measurement of the efficiency of transgenic production is the percentage of transgenic mice obtained from the number of animals born (or weaned). Such a measurement, however, does not reflect the efficiency of all the steps in transgenic production. Since every step influences the cost and effort required in producing transgenic mice, it is important to evaluate all contributing factors.

Each measurement of the efficiency of transgenic production takes into account the influence of a different subset of all the strain-dependent factors. Evaluation of transgenic production efficiency as the percentage of eggs injected measures a wider range of the processes, but still does not reflect the efficiency of embryo production (female response to superovulation and male fertility). A strong argument for expressing the efficiency of transgenic production with respect to a defined experimental day (15 females superovulated, all fertilized diploid eggs used), or to the number of eggs injected, comes from our finding that there is no significant difference in the percentage of transgenics produced per animal born between the four genetic backgrounds tested. This suggests that the frequency of DNA integration is the similar in different strains. However, when the efficiency of transgenic mouse production is expressed as a percentage of eggs injected, a significant difference was seen between all four genetic backgrounds. Thus, expressing transgenic production efficiency as a percentage of the eggs injected for the same strain is a more informative approach for comparing the results obtained by different laboratories or facilities. Calculating the percentage of transgenic mice obtained from the number of mice born, however, can be an important parameter, if it is expected that a transgene could cause lethality.

Using as an efficiency measure the number of transgenics obtained per standardized injection day or per total number of superovulated donor females used (see Materials and methods) allows the influence of all factors to be taken into account and presents a very good and simple tool for quick evaluation. If a core Transgenic Facility has a charge based on each injection day, it is also a good tool to evaluate expenses. However, if the production efficiency appears suboptimal, then the other measurements are useful for pinpointing where the problem is. Finally, one additional aspect that should be considered is the sensitivity of the genotyping assay. Since a good proportion of transgenics can be mosaic, it may be desirable to be able to detect weak mosaic transgenics and this may require PCR rather than Southern blot analysis. Depending on the genotyping procedure the true transgenic production efficiency rate of the facility might be higher than recorded. We have found this to be of particular importance for BAC transgenics in which the transgene copy number is low (A.L.J., unpublished observations).

One factor that is not strain dependent is the amount of the DNA injected into an egg. When more DNA is injected, it is often found that fewer pups are born, but a higher percentage of transgenics is obtained per animal born. We have observed as many as 70% transgenics using this measure. However, the total number of transgenic mice obtained from one experimental day can be lower when a higher concentration of DNA is injected.

Quality of transgene DNA has a major influence on transgenic production

We found that DNA transgenes purified according to the same protocol in some cases produced different efficiencies of transgenic production. We think this likely reflects a difference in the purity of the DNA, since the lower efficiencies were associated with a high rate of egg lysis and 1-cell blocks. Toxicity of DNA can be a major cause of variability in transgenic production efficiencies, when eggs from one donor strain are used. Such toxicity could result from such things as traces of phenol or ethanol, the presence of bacterial endotoxins, or from particles.

Our preliminary results (13 experiments with ~2000 eggs) using supercoiled BAC DNA injected into FVB/N eggs showed that injection of BAC DNA can produce nearly as many transgenic mice per egg injected (~2%) as plasmid-based constructs (~3%) with the same mouse strain, indicating that the size and form of a DNA construct can have little influence on the overall efficiency of transgenic production.

The key therefore is the purity of the DNA sample. For BAC constructs it is important to add polyamines to produce supercoiled DNA (Montoliu et al., 1995). Even with this BAC DNA purification method, we found that the number of 1-cell blocks was three times higher when BAC DNA was injected into zygotes (~12%) compared to plasmid-based constructs (~4%) for the same strain. Based on this, it is likely best to choose a strain with a low percentage of 1-cell blocks for making BAC transgenics.

Time of embryo transfer influences transgenic production efficiency only with some strains

There are a number of advantages of performing the embryo transfer procedure on the day after DNA injection. For instance, it allows time for rest after an afternoon of DNA injections and before the delicate procedure of transferring embryos into the oviducts of foster mothers is performed. Such timing also allows for a more precise estimate of the number of recipients that is needed for an experiment, based on the number of eggs that are actually injected. Furthermore, the number of embryos that block at the 1-cell stage can be determined, which can pinpoint problems with a particular DNA sample. However, for some strains it is better to do the embryo transfer on the same day as the DNA injection. Significantly, we found that SW eggs undergo a higher percentage of 1-cell blocks than other strains (Table 2) when they are cultured overnight after DNA injection, and that by transferring the eggs on the same day as the DNA injection this limitation was overcome (Table 4).

FVB/N mice produce the highest efficiency of transgenic production

Making transgenic founder animals in a defined inbred genetic background can be critical for experiments in which the genetic background is expected to influence the phenotype. The FVB/N strain is the most commonly used inbred strain for transgenic production because of its superior reproductive performance and prominent pronuclei, which facilitates microinjection of DNA (Taketo et al., 1991). In addition, in our hands FVB/N eggs develop in a synchronized manner. In our comparison of four strains, FVB/N embryos were found to have the highest efficiency of transgenic production when calculated either as a percentage of the eggs injected, eggs transferred, or number of transgenic mice produced per experimental day. We observed the previously reported (Taketo et al., 1991) high tolerance of FVB/N embryos for manipulation as reflected by the low number of eggs that lyzed or blocked at the 1-cell stage after injection. In general, we also noted a small range of variation with this strain, although the response of FVB/N females to superovulation was not always consistent (a range of 90–250 eggs were produced per experimental day). Of importance, egg production appeared to be particularly sensitive to the age of the donor, with 5-week-old females responding less well to superovulation than 4-week-old females.

There are some limitations to using FVB/N mice for phenotype studies, because they harbor two known mutations. One mutation causes retinal degeneration due to insertion of a provirus into the *Pde*b gene, which encodes the ß subunit of cGMP phosphodiesterase (Bowes et al., 1993; Gimenez & Montoliu, 2001). The mutation ($Pdeb^{rd1}$, previously *rd*) results in postnatal rod photoreceptor degeneration that causes severe visual impairment (FVB/N are functionally blind). One substrain maintained by the APA at NCI-FCRDC, FVB/NCr, also is afflicted by a neuroendocrine syndrome ('Space cadet' syndrome). This syndrome seems to have arisen within the past six years (Hsiao et al., 1995; Ward et al., 2000) and leads to development of neuronal necrosis in the brain and liver, with associated behavioral changes.

B6D2/F1 hybrid intercrosses produce a large number of eggs, but they are prone to lysis

Hybrid mouse lines have been used extensively for generating transgenic mice because of their superior breeding performance and efficiency of transgenic production. Typically, one of the parental inbred strains is C57BL/6 and F1 parents are used because they show hybrid vigor. The main advantage we found of using B6D2 intercrosses to produce eggs was that the superovulated females produced more eggs than the other three strains analyzed. A disadvantage of the F2 hybrid eggs is that they contain smaller pronuclei than FVB/Ns. Like FVB/N eggs, the B6D2/F2 eggs develop in a synchronized manner. However, B6D2/F2 egg survival after injection is lower than that for FVB/N due to a higher percentage of egg lysis after DNA injection.

Injected SW eggs block at the 1-cell stage at a high rate

For experiments in which the genetic background is not important (for example analysis of gene regulatory

elements), outbred strains (for example SW, CD-1, ICR) can be used to produce transgenics. However, we found that SW mice produce less transgenic animals per experimental day than FVB/N or hybrids (Table 5). The cost per transgenic mouse is therefore not lower using outbred SW mice than inbred FVB/N. The key factor that lowers the overall efficiency of transgenic production with SW mice is a high rate of 1-cell blocks. We found that SW zygotes injected with DNA blocked at the 1-cell stage four times more often than the other strains. Since we found that non-injected SW eggs cultured overnight did not block at the 1-cell stage at such a high rate (< 2% v.s. ~18% for injected), SW eggs are not the best choice for microinjection of DNA, such as BAC transgenes, which cause excessive egg damage.

C57BL/6 eggs produce fewer pups and transgenics than other strains

C57BL/6 mice have been extensively characterized genetically and are popular in many research studies. We were therefore interested in determining the optimal conditions for producing transgenics with C57BL/6 eggs. As discussed above, the percentage of transgenics produced from the number of mice born was not significantly different between C57BL/6 and the other three strains. Despite this, however, nearly every aspect of transgenic production was found to be less efficient, or more difficult, with C57BL/6 mice than with FVB/N or hybrid mice. C57BL/6 mice are poor breeders and the mothers often abandon their pups. Since C57BL/6 eggs contain granular pigmentation, their pronuclei are less visible and are more difficult to inject. The skill of the person doing the DNA injection can therefore play a critical role in the efficiency of transgenic production for C57BL/6 mice than for others. We also found that C57BL/6 preimplantation embryos often develop slower than other embryos, requiring that the DNA injections be performed later in the afternoon or the superovulation schedule be shifted ahead of the other strains. In addition, we found that C57BL/6 embryos develop asynchronously. Despite these limitations, C57BL/6 eggs tolerate DNA injection and embryo transfer well. Egg survival (Table 1) was similar to that for hybrids and better than for SW. However, the ability of C57BL/6 eggs to continue to develop to term after implantation was impaired compared to the other three strains, resulting in a low number of pups being born. Significantly, our finding that C57BL/6 transgenic mice can be produced at a frequency of ~2 transgenics per experimental day shows that it is worthwhile making transgenics in C57BL/6 eggs, rather than using hybrid mice and backcrossing to obtain an inbred background.

We performed a limited number of injections into yet another inbred strain, 129S6/SvEv (776 eggs injected; data not shown). We obtained a low (below 1%) percentage of transgenics from injected eggs (see Table 5 for other strains). The low efficiency seemed to result from poor egg survival after DNA injection (a high number of 1-cell blocks), and a low number of fetuses surviving to birth. Nevertheless, it is possible to produce transgenics with 129S6/SvEv mice.

Conclusion

Our analysis of the factors that influence transgenic production in four different strains of mice have uncovered the key steps that should be considered when making transgenics in a new strain, and has identified strain-dependent factors for each of the four strains analyzed. We have shown that the number of transgenics produced per defined experimental day, or per number of superovulated donors, is the best measurement of the transgenic production efficiency to use when comparing efficiencies in different facilities, because it takes into account all aspects of the production. Transgenic production efficiency expressed as the percentage of the transgenics obtained from the number of eggs injected is a good measure for pinpointing problems, although it does not take into consideration embryo production. By comparing the performance of four mouse strains at all steps of transgenic production, we have been able to modify the procedure for SW and C57BL/6 mice to overcome strain-dependent characteristics that decrease the overall efficiency of transgenic mouse production.

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Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter

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ABSTRACT **Promoters whose temporal activity can be directly manipulated in transgenic animals provide a tool for the study of gene functions *in vivo*. We have evaluated a tetracycline-responsive binary system for its ability to temporally control gene expression in transgenic mice. In this system, a tetracycline-controlled trans-activator protein (tTA), composed of the repressor of the tetracycline-resistance operon (*tet* from *Escherichia coli* transposon Tn*10*) and the activating domain of viral protein VP16 of herpes simplex virus, induces transcription from a minimal promoter ($P_{hCMV^{*}\text{-}1}$; see below) fused to seven *tet* operator sequences in the absence of tetracycline but not in its presence. Transgenic mice were generated that carried either a luciferase or a β-galactosidase reporter gene under the control of $P_{hCMV^{*}\text{-}1}$ or a transgene containing the tTA coding sequence under the control of the human cytomegalovirus immediate early gene 1 (hCMV *IE1*) promoter/enhancer. Whereas little luciferase or β-galactosidase activity was observed in tissues of mice carrying only the reporter genes, the presence of tTA in double-transgenic mice induced expression of the reporter genes up to several thousand-fold. This induction was abrogated to basal levels upon administration of tetracycline. These findings can be used, for example, to design dominant gain-of-function experiments in which temporal control of transgene expression is required.**

Functions of mammalian gene products in development and oncogenesis have been defined by their actions in dominant gain-of-function experiments in transgenic animals (1). The transgenes in these experiments are controlled by either tissue-specific or ubiquitously expressed promoters. Their temporal and spatial expression patterns are dependent upon the characteristics of the promoters employed. However, numerous questions require control over timing and tissue-specific expression of a transgene.

Although several inducible systems have been established in transgenic mice, all have limitations. In the classic binary systems, the target gene is silent and can be activated upon crossing in a transgene that encodes either a trans-activator or a recombinase (2–4). In such systems, the temporal activity of the target gene is dependent on the expression pattern of the effector molecule (trans-activator or recombinase), and it cannot be directly regulated by changing experimental conditions. In systems based on environmental signals such as steroid hormones or heavy metal ions, gene expression can be modulated (for review, see ref. 5). However, generalized physiologic or toxic effects from the inducing chemicals and high basal-transcriptional activity from the promoters limit their utility. Finally, tissue-specific and hormone-inducible promoters, such as the long terminal repeat of mouse mammary tumor virus or the whey acidic protein gene promoter, direct gene expression to only a few selected tissues, and the timing of gene expression is primarily controlled by endogenous hormone levels (6, 7).

Yet another approach to control gene expression has been to adopt well-characterized regulatory systems from *Escherichia coli* for use in mammalian cells (8). Transgenic systems based on the *lac* operon have proven inadequate because of inefficient induction levels and kinetics (5). However, the development of a regulatory circuit based on the tetracycline-resistance operon *tet* from *E. coli* transposon Tn*10* opened a new approach to controlling transgene expression (9, 10). In this system, a fusion tetracycline-controlled trans-activator protein (tTA) composed of the *tet* repressor and the activating domain of viral protein VP16 of herpes simplex virus strongly activates transcription from $P_{hCMV^{*}\text{-}1}$, a minimal promoter from human cytomegalovirus (hCMV) fused to *tet* operator sequences. The tTA binds to the *tet* operator sequences in the absence of tetracycline but not in its presence. This results in repression of transcription upon introduction of tetracycline. In the animal, tetracycline derivatives are readily absorbed and broadly distributed to different tissues with minimal toxicity at the concentration needed to regulate the activity of the synthetic promoter (11).

In this study we have evaluated the tetracycline-responsive regulatory system as a means to temporally regulate transgene expression in animals. The luciferase reporter gene was used as a sensitive measure of expression levels in whole tissues, and the β-galactosidase gene was used to monitor expression at the single cell level *in situ*.

MATERIALS AND METHODS

Generation of the Transgenes and Transgenic Mice. The tTA-encoding sequence contained in plasmid pUHG15-1 (P.G. and H.B., unpublished data) is under the control of the hCMV *IE1* promoter/enhancer ("hCMV-tTA gene") and is flanked at the 3′ end by the rabbit β-globin intron and a poly(A) signal. Plasmid pUHC13-3 containing the luciferase gene has been described (9). The β-galactosidase reporter gene containing a nuclear localization signal was constructed as follows: an *Xba* I–*Bgl* II fragment, containing the nuclear β-galactosidase structural gene, an intron, and a poly(A) signal, was excised from plasmid pNlacF (12) and cloned into a plasmid containing $P_{hCMV^{*}\text{-}1}$. The β-galactosidase-encoding transcription unit was separated from the vector with *Xho* I and *Bgl* II and isolated as a 4.3-kb fragment from an agarose gel by using electroelution. The hCMV-tTA gene was isolated

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Abbreviations: rlu, relative light unit(s); tTA, tetracycline-controlled trans-activator; hCMV, human cytomegalovirus.

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as a 2.7-kb *Xho* I–*Pfl*mI fragment, and the luciferase reporter gene was isolated as a 3.1-kb *Xho* I–*Eae* I fragment.

The DNA fragments were injected into fertilized eggs at a concentration of ≈5 ng per μl. Transgenic mice were generated according to standard procedures, and founder mice were analyzed by using the PCR and Southern hybridization. The tTA-encoding transgene was identified by using primers corresponding to the hCMV promoter from −50 to −33 (5′-GGC GTG TAC GGT GGG AGG-3′) and sequences encoding the *tet* repressor (5′-GCA AAA GTG AGT ATG GGT CC-3′). The resulting PCR product was 280 bp in size. The reporter genes were identified with primers corresponding to the hCMV promoter (see above) and the luciferase gene (5′-GCA ATT GTT CCA GGA ACC AGG GCG-3′) or the nuclear localization signal of the β-galactosidase gene (5′-CGG GAT CCC CCA TGC TCC CC-3′). The PCR product for the luciferase gene was 320 bp long and that for the β-galactosidase transgene was 269 bp long. Three types of transgenic mice were generated: mice that carried the hCMV-tTA gene, the luciferase reporter gene, or the β-galactosidase reporter gene containing sequences encoding a nuclear translocation signal.

Administration of Tetracycline. Slow-release tetracycline pellets (Innovative Research of America) were implanted subcutaneously in the shoulder region using a trochar according to the manufacturer's directions. These pellets released 0.7 mg of tetracycline hydrochloride per day. All pellets were kept in place for 7 days before levels of transgene expression were measured. Transgene expression following tetracycline withdrawal was measured 7 days after pellet removal. Tetracycline pellets were given to five females 1–4 days prior to mating. All females became pregnant, and a total of 50 normal pups were delivered. No toxicity from the tetracycline was seen.

Analysis of Luciferase and β-Galactosidase Activities. To analyze luciferase activity, mice were killed by cervical dislocation, and tissue samples were homogenized by using a Polytron in lysis buffer containing 25 mM glycylglycine, 15 mM Mg_2SO_4, 2 mM EDTA, 1 mM dithiothreitol, and 1% Triton X-100. The homogenate was centrifuged for 5 min at 12,000 rpm (14,000 × *g*), and 100 μl of the supernatant was added to 350 μl of assay buffer (25 mM glycylglycine, 15 mM Mg_2SO_4, 5 mM ATP). Luciferase activity was measured using a Berthold Lumat luminometer (Berthold, Germany) after the injection of 100 μl of a 0.05 mM luciferin solution. The protein concentration of the homogenate was determined by using the Bradford assay (Pierce Coomassie protein assay). Luciferase activities were calculated as relative light units (rlu) per mg of total cellular protein.

β-Galactosidase activity was assayed in whole-tissue samples or in frozen sections. To analyze activity in whole-tissue samples, 5-mm cubes of selected tissues were fixed in 2% paraformaldehyde and 0.02% glutaraldehyde in phosphate-buffered saline (PBS) for 1 hr and then rinsed twice in PBS. Staining for β-galactosidase activity was done at 30°C in a solution containing 0.1% 4-chloro-5-bromo-3-indolyl β-D-galactopyranoside, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% Nonidet P-40, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6{\cdot}6H_2O$. After staining, the specimens were embedded in paraffin, 10-μm sections were cut and counterstained with eosin or nuclear fast red, and an examination for blue-colored nuclei was conducted. To analyze β-galactosidase activity during embryogenesis, embryos were fixed for 12 hr at 4°C in PBS containing 1% formaldehyde, 0.2% glutaraldehyde, 0.2% Nonidet P-40, and 2.5 mM deoxycholic acid. Embryos were then bisectioned and stained as described above.

RESULTS

Generation of Transgenic Mice. Three types of transgenic mouse lines were generated: reporter mice containing either the luciferase or the β-galactosidase transcription unit and mice carrying the hCMV-tTA gene. The hCMV *IE1* promoter/enhancer was chosen because it is expressed in a broad spectrum of tissues in transgenic mice (13, 14). The reporter genes, encoding either luciferase or the bacterial β-galactosidase, were under the control of $P_{hCMV*-1}$. Whereas the luciferase reporter gene permitted rapid and sensitive analysis of overall transgene expression in selected organs, the β-galactosidase reporter gene enabled us to specifically identify the expressing cell types. A nuclear localization signal in the transgenic β-galactosidase allowed us to easily distinguish it from endogenous cytoplasmic β-galactosidase activity (12). From the six founder animals carrying the hCMV-tTA gene, five (TA1–TA5) were used to establish lines. From the 13 founder mice carrying the luciferase gene, 6 (LU1–LU6) were used to establish lines, and lines were established from the 4 founder animals (G1–G4) carrying the β-galactosidase gene. Double-transgenic mice carrying the hCMV-tTA gene and one of the two reporter genes were generated through cross breeding.

The Luciferase and β-Galactosidase Genes Were Activated by tTA. Basal expression levels of the luciferase gene and the magnitude of induction by tTA were most thoroughly evaluated in the thigh muscle, thymus, and tongue of the trans-

Table 1. Activation of the luciferase gene in tissues of single transgenic mice from line LU5 and in tissues from double-transgenic lines TA1/LU5, TA2/LU5, TA4/LU5, and TA5/LU5

Tissue	Luciferase activity, rlu/mg of total cellular protein (no. of animals analyzed)				
	LU5	TA1/LU5	TA2/LU5	TA4/LU5	TA5/LU5
Thigh					
− Tc	340 ± 160 (19)	10,400 ± 3,100 (4)**	14,800 ± 6,700 (7)*	32,300 ± 17,300 (9)*	53,300 ± 18,600 (4)*
+ Tc	390 ± 150 (7)	410 ± 290 (4)	90 ± 25 (7)	180 ± 110 (14)	1,500 ± 700 (4)
Post-Tc	ND	ND	ND	43,100 ± 6,000 (3)*	ND
Thymus					
− Tc	210 ± 140 (19)	6,000 ± 2,200 (4)**	11,400 ± 6,000 (7)*	5,300 ± 2,800 (9)*	1,800 ± 650 (4)*
+ Tc	230 ± 160 (7)	100 ± 30 (4)	170 ± 110 (7)	200 ± 140 (14)	290 ± 160 (4)
Post-Tc	ND	ND	ND	3,700 ± 1,000 (3)*	ND
Tongue					
− Tc	560 ± 210 (19)	26,000 ± 21,500 (4)**	12,900 ± 6,800 (7)*	27,200 ± 17,000 (9)**	45,700 ± 23,700 (4)*
+ Tc	520 ± 190 (7)	240 ± 140 (4)	180 ± 130 (7)	4,900 ± 3,800 (14)	1,300 ± 490 (4)
Post-Tc	ND	ND	ND	37,700 ± 8,100 (3)*	ND

Luciferase activities were measured in animals that had not been treated with tetracycline (−Tc), in those that had been treated with tetracycline pellets (+Tc), and in those whose tetracycline pellets had been removed 7 days earlier (post-Tc). ND, not determined. *, The difference of activity in the absence and presence of tetracycline (or in the absence of tetracycline and after removal of the tetracycline pellet) yielded a value of $P < 0.01$; **, the difference of activity in the absence and presence of tetracycline yielded a value of $P < 0.05$.

genic reporter line LU5. The basal luciferase activity in these tissues from 19 mice from line LU5 is reported in Table 1. In most mice, basal activity was close to background levels. On occasion, basal luciferase activities of up to several thousand rlu/mg of protein were measured in tongue and thymus. This suggests that there is some heterogeneity of transgene expression within a single integration site. In several mice, basal luciferase activity was analyzed in up to eight additional tissues (Table 2). The ability of the luciferase transgene in line LU5 to respond to activation by tTA was evaluated after breeding this line into the five trans-activator lines. No luciferase activity was measured in any tissue of mice transgenic for the LU5 and the TA3 locus (data not shown). This suggests that the line TA3 does not produce sufficient tTA to activate expression of the luciferase gene. The other four trans-activator lines produced sufficient tTA to activate the luciferase gene (Table 1). Trans-activation was observed in most tissues. The pattern of activity in the different tissues was similar to those reported for an hCMV *IE1*-CAT transgene (13) and an hCMV *IE1*-neo transgene (14). Levels of luciferase activity in the liver were low even in the presence of the tTA. This can be attributed to the low transcriptional activity of the hCMV *IE1* enhancer in liver cells of transgenic mice (13, 14).

Since endogenous DNA sequences in the vicinity of integration sites can exert strong position effects on the expression of transgenes, we compared the extent of induction by a single trans-activator line with three different luciferase reporter lines. Trans-activator line TA5 was bred into lines LU1, LU5, and LU6, and the magnitude of luciferase activity was measured. The tTA strongly activated the luciferase reporter gene in several tissues of all three lines (Table 2). The pattern of activation was similar in all three combinations. Specifically, expression in tongue, thigh muscle, and skin could be activated at least 100-fold. No activation of the luciferase gene was observed in the liver. The luciferase gene in an additional line did not respond to trans-activation (data not shown), suggesting that the reporter transgene was silent at this integration site.

The β-galactosidase reporter gene allowed us to analyze induction on a single-cell level. The four lines of mice carrying the β-galactosidase transgene were bred into trans-activator lines. No β-galactosidase activity was observed in mice from any of the four lines that contained only the β-galactosidase gene (data not shown). Blue-stained nuclei were observed in the thigh muscle, tongue, and seminal vesicles of mice carrying both a β-galactosidase reporter and a trans-activator gene from several combinations of adult double-transgenic mice. In all combinations tested, not all nuclei were blue, suggesting that only a subset of cells expressed both transgenes (Fig. 1). Blue-stained nuclei were less consistently found in the thymus, heart, kidney, and cerebrum from double-transgenic mice (data not shown). The tissues (tongue and thigh muscle) that demonstrated β-galactosidase activity in nearly all combinations tested were the same tissues that demonstrated high levels of luciferase activity. Activation of the β-galactosidase reporter gene by tTA was also analyzed during embryonic development. While blue staining was restricted to the spinal ganglia in day 11.5 postcoital embryos (data not shown), it was detectable in several embryonic tissues at postcoital day 16.5 (Fig. 2*B*). Particularly strong expression was seen in the nasal region, pituitary, choroid plexus, thymus, and pancreas of whole embryos. However, tissue sections revealed that not all nuclei were blue in these areas (Fig. 2*C* and data not shown). This nonuniform staining pattern was similar to that observed in sections from adult tissues (Fig. 1).

Luciferase Gene Activity Was Abrogated in the Presence of Tetracycline. To inhibit expression from the reporter genes, slow-release tetracycline pellets were implanted into mice transgenic for both the tTA gene and the luciferase reporter gene. Luciferase activities in thigh muscle, thymus, and tongue were measured after 1 week. Basal levels of luciferase activity were found in all double-transgenic mice receiving tetracycline (Table 1). This illustrates that tetracycline inactivated tTA in transgenic mice. Placebo pellets did not reduce luciferase gene activity (data not shown).

DISCUSSION

We have demonstrated that the tetracycline-responsive promoter $P_{hCMV^{*}\text{-}1}$ has low basal activity in most tissues of transgenic mice. In double-transgenic mice that synthesize the tetracycline-responsive trans-activator tTA, $P_{hCMV^{*}\text{-}1}$ was strongly activated in many tissues. The induction of gene expression was abrogated by the administration of standard therapeutic doses of tetracycline. No toxicity was observed from the exposure to tetracycline. This inducible promoter

Table 2. Activation of the luciferase target gene in transgenic lines LU1, LU5 and LU6, and induction by the tTA transcription factor from line TA5

Tissue	Luciferase activity, rlu/mg of protein		Fold induction	Luciferase activity, rlu/mg of protein		Fold induction	Luciferase activity, rlu/mg of protein		Fold induction
	LU1	LU1/TA5		LU6	LU6/TA5		LU5	LU5/TA5	
Tongue	110	14,000	130	50	116,000	2300	590	45,700	80
Thigh	240	39,500	160	130	7,700	60	350	53,300	150
Liver	20	80	4	10	120	12	20	50	2
Thymus	40	1,400	35	10	8,100	800	220	1,800	8
Heart	50	400	8	110	1,600	15	20	12,300	600
Skin	110	6,700	60	9700	267,000	30	350	73,000	200
Duodenum	15	150	10	2800	21,000	7	10	1,200	120
Colon	80	800	10	170	9,000	55	100	600	6
Whole brain	700	750	1	20	1,600	80	70	500	7
Lung	100	30	0	20	1,500	75	300	300	1
Spleen	20	1	0	350	57,000	160	130	10,700	80
Kidney	10	170	17	10	2,900	290	50	400	8
Seminal vesicle	ND	ND	ND	80	404,000	5000	1000	1,323,000	1300
Testes	ND	ND	ND	1300	2,600	2	360	23,000	64
Uterus	1	470	470	ND	ND	ND	ND	300	ND
Ovary	1	90	90	ND	ND	ND	ND	1,900	ND
Mammary gland	50	1	0	ND	ND	ND	ND	ND	ND

ND, not determined.

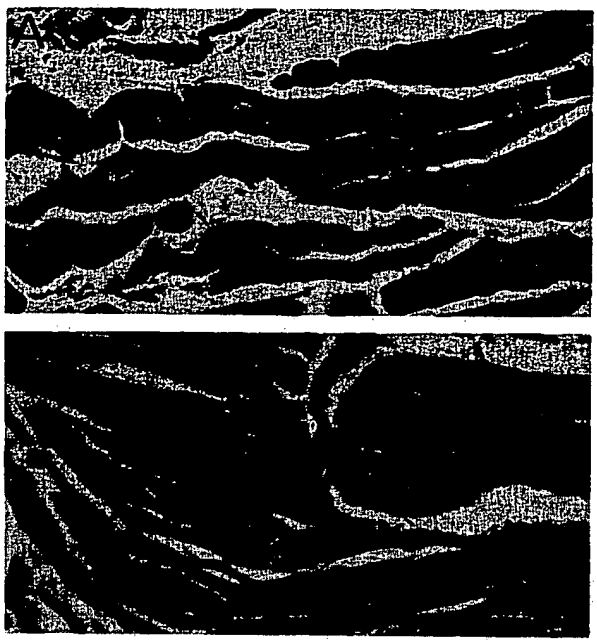

FIG. 1. β-Galactosidase activity in adult tissue sections from thigh muscle (*A*) and tongue (*B*) from an adult mouse carrying the G2 line β-galactosidase and TA1 transgenes. Cells with blue nuclei expressed the β-galactosidase gene.

system can therefore provide temporal control over gene expression in transgenic animals. It has been shown recently that the same system is functional in transgenic tobacco plants (15).

Basal Gene Activity. An essential feature of any inducible system is low promoter activity in the inactive state. Basal transcriptional activity in the *tet* repressor/VP16 system is dependent upon the promoter elements of the target gene. The activity of the $P_{hCMV*-1}$ was examined by using both the luciferase and β-galactosidase reporter genes. No measurable β-galactosidase activity was seen in mice carrying only the β-galactosidase transgene. However, measurable levels of luciferase activity were occasionally observed in certain tissues from some mice containing only the luciferase transgene. The fact that basal expression from the β-galactosidase gene was not seen is probably due to the greater sensitivity of the luciferase assay. The sporadic occurrence of measurable levels of luciferase in some mice indicates that there can be transcriptional activity from the hCMV *IE1* core promoter (16, 17). A core promoter containing only a TATA box may provide lower baseline activity (18). However, this suggestion will have to be tested to determine if such a skeletal promoter can be activated when embedded in chromatin.

Inducibility and Repression of Transgenes. Binding of the tTA transcription factor to the *tet* operator sequences in the promoter of the target gene activates transcription. High activation levels can be achieved even at low concentrations of tTA (9). The fact that tTA is capable of activating transcription of target genes in several independent chromosomal loci shows that the trans-activator can gain access to bacterial control elements packaged into chromatin in differentiated tissues. Trans-activator protein produced by four independent lines of transgenic animals activated luciferase target genes to a similar degree. This may indicate that position effects do not have a dramatic influence on the magnitude of target gene activation. Activity of the luciferase gene in double-transgenic mice was abrogated by administering tetracycline. The therapeutic levels of tetracycline released from the subcutaneously placed pellets were enough to interfere with binding of tTA to the *tet* operator sequences of $P_{hCMV*-1}$. The effect was reversible after removal of the tetracycline pellet. Specific evaluation of the kinetics of repression of gene expression following tetracycline administration or release of repression after tetracycline withdrawal was not made in this study. However, we can state that luciferase activity in double-transgenic mice was fully repressed after 7 days of tetracycline administration. Induction of gene expression followed withdrawal of tetracycline.

Variability and Mosaicism. Expression of the luciferase gene in mice carrying also the hCMV-tTA gene varied between animals from any given line and even between littermates. This variability may be inherent in the hCMV *IE1*

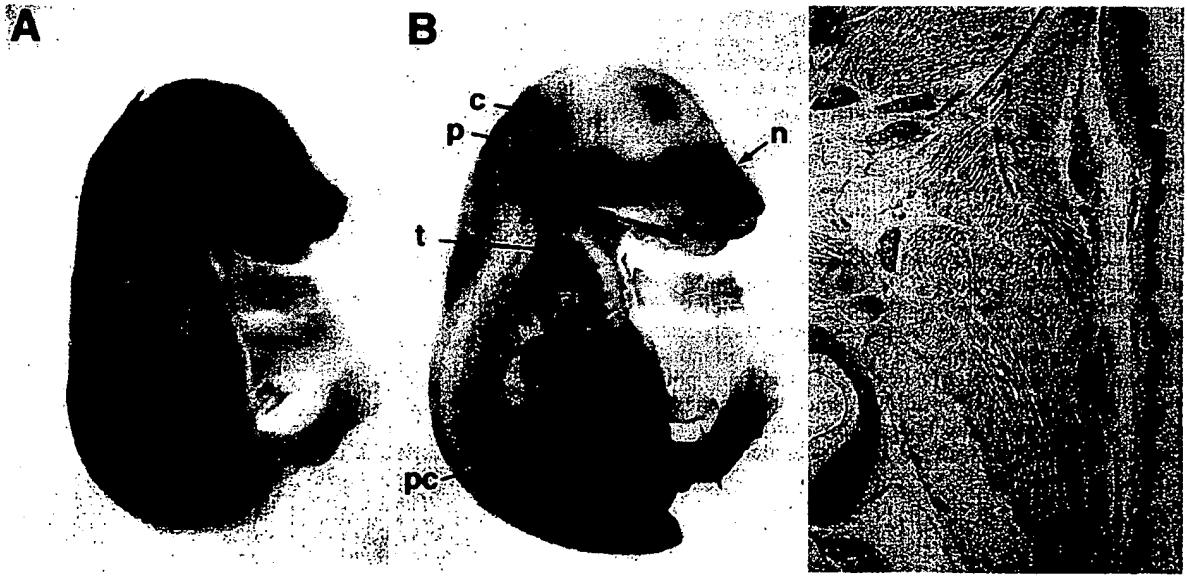

FIG. 2. β-Galactosidase activity in bisectioned day 16.5 postcoital embryos. (*A*) Embryo carrying the G4 line β-galactosidase transgene. (*B*) Embryo carrying the G4 and TA4 transgenes. c, Chloroid plexus; n, nasal region; p, pituitary; pc, pancreas; t, thymus. The blue staining of intestinal tissue is due to endogenous β-galactosidase activity. (*C*) Section from the neck region of the embryo shown in *B*. Cells with blue nuclei expressed the β-galactosidase gene.

promoter/enhancer used to direct expression of the trans-activator. Such variability was also observed in littermates carrying a hCMV *IE1*-CAT transgene (13). Histological analyses for β-galactosidase activity revealed mosaicism. The number of cells expressing the transgene was only a subset of the cells expected to stain in that site, a pattern reminiscent of position-effect variegation (19). Although a possibility, mosaicism cannot be attributed simply to the use of the β-galactosidase reporter gene as this gene has been expressed homogeneously in embryonic, fetal, and adult tissues (20). Mosaicism has been observed with other transgenes (12, 14), and even some endogenous genes (21), and is referred to as incomplete penetrance (12). This stochastic pattern of gene expression may reflect the activity of certain endogenous genes (21) and also may be the cause of the variable penetrance of defects observed in null mutant mice (22, 23).

Other Inducible Systems. Other inducible promoter systems have not offered the degree of control presented by this strategy. Similar to the system reported here, two previously described binary systems consist of a silent target gene that is induced by constitutive or regulated trans-activators (2, 3). In the third reported system, a silent target gene is activated by a site-specific recombinase (4). However, in contrast to the system described here, transgene activity in these binary systems is regulated by the transcription pattern inherent to the promoter controlling the trans-activator gene. No additional manipulation is possible.

A system that uses the *tet* repressor to inhibit gene transcription is another approach to controlling gene activity. In these systems *tet* repressor molecules bind to *tet* operator sequences located at the transcriptional start site and block gene transcription in the absence of tetracycline. In the presence of tetracycline binding of the tet repressor to the *tet* operator is greatly reduced and transcription is activated (24). Repression of transcriptional activity to basal levels has been achieved in the presence of ≈500,000 tet repressor molecules per cell. Such a concentration can only be obtained with strong promoters, such as the 35S promoter from the cauliflower mosaic virus (24). Therefore, it may be difficult to achieve a repression of the transgene with housekeeping or standard tissue-specific promoters.

The induction of transgenes through the withdrawal of tetracycline can have specific advantages for some experiments. For example, when analyzing the roles of oncogenes, growth factors, or tumor suppressor genes on tumor formation, a long period of gene activation may be required (25–28). If a tetracycline-responsive promoter is used to control oncogene expression, it may be convenient to have the animals off tetracycline during this time.

In conclusion, the tetracycline regulatory system can provide temporal control of transgene expression. It should be useful for experiments designed to address certain biological questions in transgenic animals. For example, temporal control of the induction of growth modulators, oncoproteins, and other proteins participating in developmental processes could provide further definition to their roles in normal growth and tumorigenesis. The effects of expressing potentially deleterious genes can be studied, since these genes can be rendered inactive by using tetracycline. Alternatively, the system can be combined with one of the site-specific recombinases (4) and used to delete genes at specific time points during development.

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